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PATENT APPLICATION
TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No. 10447-011

First Inventor or Application Identifier THOR BORGFORDE

Title RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF ...

Express Mail Label No.

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original and a duplicate for fee processing)
2. ☒ Specification [Total Pages 88]
(preferred arrangement set forth below)
- Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Micro fiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 254]
4. Oath or Declaration [Total Pages 3]
- a. ☒ Newly executed (original or copy)
 - b. ☐ Copy from a prior application (37 C.F.R. § 1.63(d))
(for continuation/divisional with Box 16 completed)
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

* NOTE FOR ITEMS 1 & 13: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY
FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT
IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

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Washington, DC 20231

5. ☐ Microfiche Computer Program (Appendix)
6. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
- a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. ☐ Assignment Papers (cover sheet & document(s))
8. ☐ 37 C.F.R. § 3.73(b) Statement of Power of Attorney
(when there is an assignee)
9. ☐ English Translation Document (if applicable)
10. ☐ Information Disclosure Statement (IDS)/PTO-1449 [Copies of IDS Citations]
11. ☐ Preliminary Amendment
12. ☐ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
13. ☒ * Small Entity Statement filed in prior application,
Statement(s) (PTO/SB/09-12) Status still proper and desired
14. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
15. ☐ Other:

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application No: 09/403,752

Prior application information: Examiner

Group / Art Unit:

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied
under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by
reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

17. CORRESPONDENCE ADDRESS

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Signature		Date	April 13, 2000

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April 13, 2000

The Commissioner of Patents & Trademarks
Washington, D.C., U.S.A. 20231

Dear Sir:

Transmitted herewith for filing is the patent application of

Inventor: **Thor Borgford**

For: **RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF CANCER, VIRAL OR PARASITIC INFECTIONS**

The following papers are also enclosed:

[X] 254 sheets of X Formal drawings _____ Informal drawings

[X] A signed declaration.

	Number Extra	Full Rate	Small Entity Rate	Total
Total Claims	39 - 20 = 19 X	\$ 18.00	\$ 9.00	\$171
Independent Claims	5 - 3 = 2 X	\$ 78.00	\$ 39.00	\$78
Multiple Claims	Flat Rate	\$260.00	\$130.00	\$0

Basic Fee = \$345.00
TOTAL FEES = \$594.00

[X] Two Verified Statements Claiming Small Entity Status.

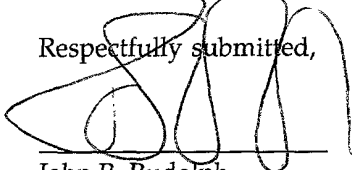
[X] The amount of \$594.00 is enclosed in our cheque No. 2860.

[X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Account No. 02-2095. This letter is enclosed in duplicate.

[X] Related application. This application is a _____ Division _____ Continuation
X Continuation-in-part of application Serial No. 09/403,752, filed October 29, 1999.

[] Convention priority is requested based on U.S. Application Serial No. _____ filed _____, under 35 USC §119(e).

Respectfully submitted,


John R. Rudolph
Registration No. 38,003

Applicant or Patentee: Thor Borgford
 Serial or Patent No.: _____
 Filed or Issued: _____
 For: Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections described in

- ☒ the specification filed herewith
☐ Application serial no. _____, filed _____
☐ patent no. _____, issued _____

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☐ no such person, concern, or organization
☒ persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME De Novo Enzyme Corporation
 ADDRESS 8081 Lougheed Highway, Burnaby, B.C. V5A 1W9, Canada
☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Thor Borgford
 Name of Inventor

X Thor Borgford
 Signature of Inventor

X APRIL 13, 2000
 Date

09551151 "041400

Applicant or Patentee: Thor Borgford

Serial or Patent No.: _____

Filed or Issued: _____

For: Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27 (e)) - SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN De Novo Enzyme Corporation

ADDRESS OF CONCERN 8081 Lougheed Highway, Burnaby, B.C. V5A 1W9, Canada

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled Ricin-Like Toxin Variants For Treatment of Cancer, Viral or Parasitic Infections by inventor Thor Borgford described in

☒ the specification filed herewith

☐ PCT application serial no. _____ filed _____

☐ patent no. _____ issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____

ADDRESS _____

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING CURTIS BRAUN

TITLE OF PERSON OTHER THAN OWNER DIRECTOR OF OPERATIONS

ADDRESS OF PERSON SIGNING 11519 140 A STREET SURREY BC CANADA V2R 1J1

SIGNATURE [Signature]

DATE APRIL 13, 2000

00447-011-10447-011

B&P File No. 10447-011

BERESKIN & PARR

UNITED STATES

**Title: RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF
CANCER, VIRAL OR PARASITIC INFECTIONS**

Inventor: THOR BORGFORD

00447-011-0000

**Title: RICIN-LIKE TOXIN VARIANTS FOR TREATMENT OF
CANCER, VIRAL OR PARASITIC INFECTIONS**

This is a continuation-in-part of U.S. patent application serial
No. 09/403,752 filed October 29, 1999.

5 **FIELD OF THE INVENTION**

The invention relates to proteins useful as therapeutics
against cancer, viral infections, parasitic and fungal infections. The proteins
contain A and B chains of a ricin-like toxin linked by a linker sequence that
is specifically cleaved and activated by proteases specific to disease-
10 associated pathogens or cells.

BACKGROUND OF THE INVENTION

Bacteria and plants are known to produce cytotoxic proteins
which may consist of one, two or several polypeptides or subunits. Those
proteins having a single subunit may be loosely classified as Type I
15 proteins. Many of the cytotoxins which have evolved two subunit
structures are referred to as type II proteins (Saelinger, C.B. in Trafficking
of Bacterial Toxins (eds. Saelinger, C.B.) 1-13 (CRC Press Inc., Boca Raton,
Florida, 1990). One subunit, the A chain, possesses the toxic activity
whereas the second subunit, the B chain, binds cell surfaces and mediates
20 entry of the toxin into a target cell. A subset of these toxins kill target cells
by inhibiting protein biosynthesis. For example, bacterial toxins such as
diphtheria toxin or Pseudomonas exotoxin inhibit protein synthesis by
inactivating elongation factor 2. Plant toxins such as ricin, abrin, and
bacterial toxin Shiga toxin, inhibit protein synthesis by directly inactivating
25 the ribosomes (Olsnes, S. & Phil, A. in Molecular action of toxins and
viruses (eds. Cohen, P. & vanHeyningen, S.) 51-105 Elsevier Biomedical
Press, Amsterdam, 1982).

Ricin, derived from the seeds of *Ricinus communis* (castor oil
plant), may be the most potent of the plant toxins. It is estimated that a
30 single ricin A chain is able to inactivate ribosomes at a rate of 1500
ribosomes/minute. Consequently, a single molecule of ricin is enough to
kill a cell (Olsnes, S. & Phil, A. in Molecular action of toxins and viruses
(eds. Cohen, P. & vanHeyningen, S.) (Elsevier Biomedical Press,
Amsterdam, 1982). The ricin toxin is a glycosylated heterodimer consisting

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of A and B chains with molecular masses of 30,625 Da and 31,431 Da linked by a disulphide bond. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y. & Tsurugi, K. J., *Biol. Chem.* 262:8128
5 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)). Once the toxin molecule consisting of the A and B chains is internalized into the cell via
10 clathrin-dependent or independent mechanisms, the greater reduction potential within the cell induces a release of the active A chain, eliciting its inhibitory effect on protein synthesis and its cytotoxicity (Emmanuel, F. et al., *Anal. Biochem.* 173: 134-141 (1988); Blum, J.S. et al., *J. Biol. Chem.* 266: 22091-22095 (1991); Fiani, M.L. et al., *Arch. Biochem. Biophys.* 307: 225-230
15 (1993)). Empirical evidence suggests that activated toxin (e.g. ricin, shiga toxin and others) in the endosomes is transcytosed through the trans-Golgi network to the endoplasmic reticulum by retrograde transport before the A chain is translocated into the cytoplasm to elicit its action (Sandvig, K. & van Deurs, B., *FEBS Lett.* 346: 99-102 (1994)).
20 Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416
25 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an
30 interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside the plant cells. The A chain is inactive in proricin (O'Hare, M. et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the

castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell. The exact mechanism of A chain release and activation in target cell cytoplasm is not known (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). However, it is known that for activation to take place the disulfide bond between the A and B chains must be reduced and, hence, the linkage between subunits broken.

Diphtheria toxin is produced by *Corynebacterium diphtheriae* as a 535 amino acid polypeptide with a molecular weight of approximately 58kD (Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Collier, R.J. & Kandel, J., *J. Biol. Chem.* 246:1496-1503 (1971)). It is secreted as a single-chain polypeptide consisting of 2 functional domains. Similar to proricin, the N-terminal domain (A-chain) contains the cytotoxic moiety whereas the C-terminal domain (B-chain) is responsible for binding to the cells and facilitates toxin endocytosis. Conversely, the mechanism of cytotoxicity for diphtheria toxin is based on ADP-ribosylation of EF-2 thereby blocking protein synthesis and producing cell death. The 2 functional domains in diphtheria toxin are linked by an arginine-rich peptide sequence as well as a disulphide bond. Once the diphtheria toxin is internalized into the cell, the arginine-rich peptide linker is cleaved by trypsin-like enzymes and the disulphide bond (Cys 186-201) is reduced. The cytotoxic domain is subsequently translocated into the cytosol substantially as described above for ricin and elicits ribosomal inhibition and cytotoxicity.

Pseudomonas exotoxin is also a 66kD single-chain toxin protein secreted by *Pseudomonas aeruginosa* with a similar mechanism of cytotoxicity to that of diphtheria toxin (Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Ogata, M. et al., *J. Biol. Chem.* 267:25396-25401 (1992); Vagil, M.L. et al., *Infect. Immunol.* 16:353-361 (1977)). *Pseudomonas* exotoxin consists of 3 conjoint functional domains. The first domain Ia (amino acids 1-252) is responsible for cell binding and toxin endocytosis, a second domain II (amino acids 253-364) is responsible for toxin translocation from the endocytic vesicle to the cytosol, and a third domain III (amino acids 400-613) is responsible for protein synthesis inhibition and cytotoxicity.

After *Pseudomonas* exotoxin enters the cell, the liberation of the cytotoxic domain is effected by both proteolytic cleavage of a polypeptide sequence in the second domain (near Arg 279) and the reduction of the disulphide bond (Cys 265-287) in the endocytic vesicles. In essence, the overall pathway to cytotoxicity is analogous to diphtheria toxin with the exception that the toxin translocation domain in *Pseudomonas* exotoxin is structurally distinct.

Class 2 ribosomal inhibitory proteins (RIP-2) constitute other toxins possessing distinct functional domains for cytotoxicity and cell binding/toxin translocation which include abrin, modeccin, volkensin, (Sandvig, K. et al., *Biochem. Soc. Trans.* 21:707-711 (1993)) and mistle toe lectin (viscumin) (Olsnes, S. & Phil, A. in *Molecular action of toxins and viruses* (eds. Cohen, P. & vanHeyningen, S.) 51-105 Elsevier Biomedical Press, Amsterdam, 1982; and Fodstad, et al. *Canc. Res.* 44:862 (1984)). Some toxins such as Shiga toxin and cholera toxin also have multiple polypeptide chains responsible for receptor binding and endocytosis.

The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains have been described (Rutenber, E. et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Bio.* 244:410-422, 1994; Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K. et al. *Nucleic Acids Res.* 13:8019 (1985)). Similarly, the genes for diphtheria toxin and *Pseudomonas* exotoxin have been cloned and sequenced, and the 3-dimensional structures of the toxin proteins have been elucidated and described (Columblatti, M. et al., *J. Biol. Chem.* 261:3030-3035 (1986); Allured, V.S. et al., *Proc. Natl. Acad. Sci. USA* 83:1320-1324 (1986); Gray, G.L. et al., *Proc. Natl. Acad. Sci. USA* 81:2645-2649 (1984); Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Collier, R.J. et al., *J. Biol. Chem.* 257:5283-5285 (1982)).

The potential of bacterial and plant toxins for inhibiting mammalian retroviruses, particularly acquired immunodeficiency syndrome (AIDS), has been investigated. Bacterial toxins such as *Pseudomonas* exotoxin-A and subunit A of diphtheria toxin; dual chain ribosomal inhibitory plant toxins such as ricin, and single chain ribosomal inhibitory proteins such as trichosanthin and pokeweed antiviral protein

have been used for the elimination of HIV infected cells (Olson et al., *AIDS Res. and Human Retroviruses* 7:1025-1030 (1991)). The high toxicity of these toxins for mammalian cells, combined with a lack of specificity of action poses a major problem to the development of pharmaceuticals incorporating the toxins, such as immunotoxins.

Due to their extreme toxicity there has been much interest in making ricin-based immunotoxins as therapeutic agents for specifically destroying or inhibiting infected or tumourous cells or tissues (Vitetta et al., *Science* 238:1098-1104(1987)). An immunotoxin is a conjugate of a specific cell binding component, such as a monoclonal antibody or growth factor and the toxin in which the two protein components are covalently linked. Generally, the components are chemically coupled. However, the linkage may also be a peptide or disulfide bond. The antibody directs the toxin to cell types presenting a specific antigen thereby providing a specificity of action not possible with the natural toxin. Immunotoxins have been made both with the entire ricin molecule (i.e. both chains) and with the ricin A chain alone (Spooner et al., *Mol. Immunol.* 31:117-125, (1994)).

Immunotoxins made with the ricin dimer (IT-Rs) are more potent toxins than those made with only the A chain (IT-As). The increased toxicity of IT-Rs is thought to be attributed to the dual role of the B chains in binding to the cell surface and in translocating the A chain to the cytosolic compartment of the target cell (Vitetta et al., *Science* 238:1098-1104 (1987); Vitetta & Thorpe, *Seminars in Cell Biology* 2:47-58 (1991)). However, the presence of the B chain in these conjugates also promotes the entry of the immunotoxin into nontarget cells. Even small amounts of B chain may override the specificity of the cell-binding component as the B chain will bind nonspecifically to galactose associated with N-linked carbohydrates, which is present on most cells. IT-As are more specific and safer to use than IT-Rs. However, in the absence of the B chain the A chain has greatly reduced toxicity. Due to the reduced potency of IT-As as compared to IT-Rs, large doses of IT-As must be administered to patients. The large doses frequently cause immune responses and production of neutralizing antibodies in patients (Vitetta et al., *Science* 238:1098-1104

(1987)). IT-As and IT-Rs both suffer from reduced toxicity as the A chain is not released from the conjugate into the target cell cytoplasm.

5 A number of immunotoxins have been designed to recognize antigens on the surfaces of tumour cells and cells of the immune system (Pastan et al., *Annals New York Academy of Sciences* 758:345-353 (1995)). A major problem with the use of such immunotoxins is that the antibody component is its only targeting mechanism and the target antigen is often found on non-target cells (Vitetta et al., *Immunology Today* 14:252-259 (1993)). Also, the preparation of a suitable specific cell binding
10 component may be problematic. For example, antigens specific for the target cell may not be available and many potential target cells and infective organisms can alter their antigenic make up rapidly to avoid immune recognition. In view of the extreme toxicity of proteins such as ricin, the lack of specificity of the immunotoxins may severely limit their
15 usefulness as therapeutics for the treatment of cancer and infectious diseases.

The insertion of intramolecular protease cleavage sites between the cytotoxic and cell-binding components of a toxin can mimic the way that the natural toxin is activated. European patent application no.
20 466,222 describes the use of maize-derived pro-proteins which can be converted into active form by cleavage with extracellular blood enzymes such as factor Xa, thrombin or collagenase. Garred, O. et al. (*J. Biol. Chem.* 270:10817-10821 (1995)) documented the use of a ubiquitous calcium-dependent serine protease, furin, to activate shiga toxin by cleavage of the
25 trypsin-sensitive linkage between the cytotoxic A-chain and the pentamer of cell-binding B-units. Westby et al. (*Bioconjugate Chem.* 3:375-381 (1992)) documented fusion proteins which have a specific cell binding component and proricin with a protease sensitive cleavage site specific for factor Xa within the linker sequence. O'Hare et al. (*FEBS Lett.* 273:200-204 (1990))
30 also described a recombinant fusion protein of RTA and staphylococcal protein A joined by a trypsin-sensitive cleavage site. In view of the ubiquitous nature of the extracellular proteases utilized in these approaches, such artificial activation of the toxin precursor or immunotoxin does not confer a mechanism for intracellular toxin

activation and the problems of target specificity and adverse immunological reactions to the cell-binding component of the immunotoxin remain.

In a variation of the approach of insertion of intramolecular protease cleavage sites on proteins which combine a binding chain and a toxic chain, Leppla, S.H. et al. (Bacterial Protein Toxins zbl.bakt.suppl. 24:431-442 (1994)) suggest the replacement of the native cleavage site of the protective antigen (PA) produced by *Bacillus anthracis* with a cleavage site that is recognized by cells that contain a particular protease. PA, recognizes, binds, and thereby assists in the internalization of lethal factor (LF) and edema toxin (ET). also produced by *Bacillus anthracis*. However, this approach is wholly dependent on the availability of LF, or ET and PA all being localized to cells wherein the modified PA can be activated by the specific protease. It does not confer a mechanism for intracellular toxin activation and presents a problem of ensuring sufficient quantities of toxin for internalization in target cells.

The *in vitro* activation of a *Staphylococcus*-derived pore-forming toxin, α -hemolysin by extracellular tumour-associated proteases has been documented (Panchel, R.G. et al., *Nature Biotechnology* 14:852-857 (1996)). Artificial activation of α -hemolysin *in vitro* by said proteases was reported but the actual activity and utility of α -hemolysin in the destruction of target cells were not demonstrated.

Hemolysin does not inhibit protein synthesis but is a heptameric transmembrane pore which acts as a channel to allow leakage of molecules up to 3 kD thereby disrupting the ionic balances of the living cell. The α -hemolysin activation domain is likely located on the outside of the target cell (for activation by extracellular proteases). The triggering mechanism in the disclosed hemolysin precursor does not involve the intracellular proteolytic cleavage of 2 functionally distinct domains. Also, the proteases used for the α -hemolysin activation are ubiquitously secreted extracellular proteases and toxin activation would not be confined to activation in the vicinity of diseased cells. Such widespread activation of the toxin does not confer target specificity and limits the usefulness of said α -hemolysin toxin as therapeutics due to systemic toxicity.

UNIVERSITY OF CALIFORNIA

A variety of proteases specifically associated with malignancy, viral infections and parasitic infections have been identified and described. For example, cathepsin is a family of serine, cysteine or aspartic endopeptidases and exopeptidases which has been implicated to
5 play a primary role in cancer metastasis (Schwartz, M.K., *Clin. Chim. Acta* 237:67-78 (1995); Spiess, E. et al., *J. Histochem. Cytochem.* 42:917-929 (1994); Scarborough, P.E. et al., *Protein Sci.* 2:264-276 (1993); Sloane, B.F. et al., *Proc. Natl. Acad. Sci. USA* 83:2483-2487 (1986); Mikkelsen, T. et al., *J. Neurosurg* 83:285-290 (1995)). Matrix metalloproteinases (MMPs or matrixins) are
10 zinc-dependent proteinases consisting of collagenases, matrilysin, stromelysins, gelatinases and macrophage elastase (Krane, S.M., *Ann. N.Y. Acad. Sci.* 732:1-10 (1994); Woessner, J.F., *Ann. N.Y. Acad. Sci.* 732:11-21 (1994); Carvalho, K. et al., *Biochem. Biophys. Res. Comm.* 191:172-179 (1993); Nakano, A. et al. *J. of Neurosurg*, 83:298-307 (1995); Peng, K-W, et al.
15 *Human Gene Therapy*, 8:729-738 (1997); More, D.H. et al. *Gynaecologic Oncology*, 65:78-82 (1997)). These proteases are involved in pathological matrix remodeling. Under normal physiological conditions, regulation of matrixin activity is effected at the level of gene expression. Enzymatic activity is also controlled stringently by tissue inhibitors of
20 metalloproteinases (TIMPs) (Murphy, G. et al., *Ann. N.Y. Acad. Sci.* 732:31-41 (1994)). The expression of MMP genes is reported to be activated in inflammatory disorders (e.g. rheumatoid arthritis) and malignancy.

In malaria, parasitic serine and aspartic proteases are involved in host erythrocyte invasion by the *Plasmodium* parasite and in
25 hemoglobin catabolism by intraerythrocytic malaria (O'Dea, K.P. et al., *Mol. Biochem. Parasitol.* 72:111-119 (1995); Blackman, M.J. et al., *Mol. Biochem. Parasitol.* 62:103-114 (1993); Cooper, J.A. et al., *Mol. Biochem. Parasitol.* 56:151-160 (1992); Goldberg, D.E. et al., *J. Exp. Med.* 173:961-969 (1991)). *Schistosoma mansoni* is also a pathogenic parasite which causes
30 schistosomiasis or bilharzia. Elastinolytic proteinases have been associated specifically with the virulence of this particular parasite (McKerrow, J.H. et al., *J. Biol. Chem.* 260:3703-3707 (1985)).

Welch, A.R. et al. (*Proc. Natl. Acad. Sci. USA* 88:10797-10800 (1991)) has described a series of viral proteases which are specifically

associated with human cytomegalovirus, human herpesviruses, Epstein-Barr virus, varicella zoster virus-I. and infectious laryngotracheitis virus. These proteases possess similar substrate specificity and play an integral role in viral scaffold protein restructuring in capsid assembly and virus maturation. Other viral proteases serving similar functions have also been documented for human T-cell leukemia virus (Blaha, I. et al., *FEBS Lett.* 309:389-393 (1992); Pettit, S.C. et al., *J. Biol. Chem.* 266:14539-14547 (1991)), hepatitis viruses (Hirowatari, Y. et al., *Anal. Biochem.* 225:113-120 (1995); Hirowatari, Y. et al., *Arch. Virol.* 133:349-356 (1993); Jewell, D.A. et al., *Biochemistry* 31:7862-7869 (1992)), poliomyelitis virus (Weidner, J.R. et al., *Arch. Biochem. Biophys.* 286:402-408 (1991)), and human rhinovirus (Long, A.C. et al., *FEBS Lett.* 258:75-78 (1989)).

Candida yeasts are dimorphic fungi which are responsible for a majority of opportunistic infections in AIDS patients (Holmberg, K. and Myer, R., *Scand. J. Infect. Dis.* 18:179-192 (1986)). Aspartic proteinases have been associated specifically with numerous virulent strains of *Candida* including *Candida albican*, *Candida tropicalis*, and *Candida parapsilosis* (Abad-Zapatero, C. et al., *Protein Sci.* 5:640-652 (1996); Cutfield, S.M. et al., *Biochemistry* 35:398-410 (1995); Ruchel, R. et al., *Zentralbl. Bakteriол. Mikrobiol Hyg. I Abt. Orig. A.* 255:537-548 (1983); Remold, H. et al., *Biochim. Biophys. Acta* 167:399-406 (1968)), and the levels of these enzymes have been correlated with the lethality of the strain (Schreiber, B, et al., *Diagn. Microbiol. Infect. Dis.* 3:1-5 (1985)).

SUMMARY OF THE INVENTION

The invention relates to novel recombinant toxic proteins which are specifically toxic to diseased cells but do not depend for their specificity of action on a specific cell binding component. The recombinant proteins of the invention have an A chain of a ricin-like toxin linked to a B chain by a synthetic linker sequence which may be cleaved specifically by a protease localised in cells or tissues affected by a specific disease to liberate the toxic A chain thereby selectively inhibiting or destroying the diseased cells or tissues. The term diseased cells as used herein, includes cells affected by cancer, or infected by fungi, or viruses, including retroviruses, or parasites.

Toxin targeting using the recombinant toxic proteins of the invention takes advantage of the fact that many DNA viruses exploit host cellular transport mechanisms to escape immunological destruction. This is achieved by enhancing the retrograde translocation of host major histocompatibility complex (MHC) type I molecules from the endoplasmic reticulum into the cytoplasm (Bonifacino, J.S., *Nature* 384: 405-406 (1996); Wiertz, E.J. et al., *Nature* 384: 432-438 (1996)). The facilitation of retrograde transport in diseased cells by the virus can enhance the transcytosis and cytotoxicity of a recombinant toxic protein of the present invention thereby further reducing non-specific cytotoxicity and improving the overall safety of the product.

The recombinant toxic proteins of the present invention may be used to treat diseases including various forms of cancer such as T- and B-cell lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate cancer, non small cell lung cancer, malaria, and diverse viral disease states associated with infection with human cytomegalovirus, hepatitis virus, herpes virus, human rhinovirus, infectious laryngotracheitis virus, poliomyelitis virus, or varicella zoster virus.

In one aspect, the present invention provides a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence is not a native linker sequence of a ricin-like toxin, but rather a synthetic heterologous linker sequence containing a cleavage recognition site for a disease-specific protease. The A and or the B chain may be those of ricin.

In an embodiment, of the invention the cleavage recognition site is the cleavage recognition site for a cancer-associated protease. In particular embodiments, the linker amino acid sequence comprises SLLKSRMVPNFN or SLLIARRMPNFN cleaved by cathepsin B; SKLVQASASGVN or SSYLKASDAPDN cleaved by an Epstein-Barr virus protease; RPKPQQFFGLMN cleaved by MMP-3 (stromelysin); SLRPLALWRSFN cleaved by MMP-7 (matrilysin); SPQGIAGQRNFN

cleaved by MMP-9; DVDERDVGRGFASFL cleaved by a thermolysin-like
MMP; SLPLGLWAPNFN cleaved by matrix metalloproteinase 2(MMP-2) ;
SLLIFRSWANFN cleaved by cathepsin L; SGVVIATVIVIT cleaved by
cathepsin D; SLGPQGIWGQFN cleaved by matrix metalloproteinase
5 1(MMP-1); KKSPGRVVGGSV cleaved by urokinase-type plasminogen
activator; PQGLLGAPGILG cleaved by membrane type 1
matrixmetalloproteinase (MT-MMP);
HGPEGLRVGFYESDVMGRGHARLVHVEEPHT cleaved by stromelysin 3
(or MMP-11), thermolysin, fibroblast collagenase and stromelysin-1;
10 GPQGLAGQRGIV cleaved by matrix metalloproteinase 13 (collagenase-3);
GGSGQRGRKALE cleaved by tissue-type plasminogen activator(tPA);
SLSALLSSDIFN cleaved by human prostate-specific antigen;
SLPRFKIIGGFN cleaved by kallikrein (hK3); SLLGIAVPGNFN cleaved by
neutrophil elastase; and FFKNIVTPRTPP cleaved by calpain (calcium
15 activated neutral protease). The nucleic acid sequences for ricin A and B
chains with each of the linker sequences are shown in Figures 2D, 35C, 3D,
4D, 5D, 6D, 16D, 17D, 34C, 36C , 37C, 38C , 39C, 40C, 41C, 42C , 43C, 44C,
45C, 46C and 47C, respectively.

In another embodiment, the cleavage recognition site is the
20 cleavage recognition site for a protease associated with the malaria
parasite, *Plasmodium falciparum*. In particular embodiments, the linker
amino acid sequence comprises QVVQLQNYDEED; LPIFGESEDNDE;
QVVTGEAISVTM; ALERTFLSFPTN or KFQDMLNISQHQ. The nucleic
nucleotide sequences for ricin A and B chains with each of the linker
25 sequences are shown in Figures 7D, 8D, 9D, 10D, and 11D.

In a another embodiment, the cleavage recognition site is
the cleavage recognition site for a viral protease. The linker sequences
preferably comprise the sequence Y-X-Y-A-Z wherein X is valine or
leucine, Y is a polar amino acid, and Z is serine, asparagine or valine. In
30 particular embodiments, the linker amino acid sequence comprises
SGVVNASCRLAN or SSYVKASVSPEN cleaved by a human
cytomegalovirus protease; SALVNASSAHVN or STYLQASEKFKN cleaved
by a herpes simplex 1 virus protease; SSILNASVPNFN cleaved by a
human herpes virus 6 protease; SQDVNAVEASSN or SVYLQASTGYGN

cleaved by a varicella zoster virus protease; or SKYLQANEVITN cleaved by an infectious laryngotracheitis virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker sequences are shown in Figures 12D, 13D, 14D, 15D, 18D, 19D, 20D, and 22D.

5 In another embodiment, the cleavage recognition site is the cleavage recognition site for a hepatitis A viral protease. In particular embodiments, the linker amino acid sequence comprises SELRTQSFSNWN or SELWSQGIDDDN cleaved by a hepatitis A virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker
10 sequences are shown in Figures 23D or 24D.

In another embodiment, the cleavage recognition site is the cleavage recognition site for a hepatitis C viral protease. In particular embodiments, the linker amino acid sequence comprises DLEVVTSTWVFN, DEMEECASHLFN, EDVVCCSMSYFN or
15 KGWRL LAPITAY cleaved by a hepatitis C virus protease. The nucleic nucleotide sequences for ricin A and B chains with each of the linker sequences are shown in Figures 30C, 31C, 32C and 33C.

In another embodiment, the cleavage recognition site is the cleavage recognition site for a *Candida* fungal protease. In particular
20 embodiments, the linker amino acid sequence is SKPAKFFRLNFN, SKPIEFFRLNFN or SKPAEFFALNFN cleaved by *Candida* aspartic protease. The nucleic nucleotide sequences for ricin A and B chains with the first linker sequence are shown in Figures 25D.

The present invention also provides a plasmid incorporating
25 the nucleic acid of the invention. In an embodiment, the plasmid has the restriction map as shown in Figures 2A, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 12A, 13A, 14A, 15A, 16A, 17A, 18A, 19A, 20A, 21A, 22A, 23A, 24A, or 25A.

In another embodiment, the present invention provides a
30 baculovirus transfer vector incorporating the nucleic acid of the invention. In particular embodiments, the invention provides a baculovirus transfer vector having the DNA sequence as shown in Figure 1.

In a further embodiment, the present invention provides a baculovirus transfer vector incorporating the nucleic acid of the invention.

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In particular embodiments, the invention provides a baculovirus transfer vector having the restriction map as shown in Figures 2C, 3C, 4C, 5C, 6C, 7C, 8C, 9C, 10C, 11C, 12C, 13C, 14C, 15C, 16C, 17C, 18C, 19C, 20C, 21C, 22C, 23C, 24C, 25C, 30A, 31A, 32A, 33A, 34A, 35A, 36A, 37A, 38A, 39A, 5 40A, 41A, 42A, 43A, 44A, 45A, 46A, or 47A. or having the DNA sequence as shown in Figure 1.

In a further aspect, the present invention provides a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking 10 the A and B chains, wherein the linker sequence contains a cleavage recognition site for a disease-specific protease (e.g., a cancer, viral, parasitic, or fungal protease). The A and/or the B chain may be those of ricin. In an embodiment, the cleavage recognition site is the cleavage recognition site for a cancer, viral or parasitic protease substantially as described above. In 15 a particular embodiment, the cancer is T-cell or B-cell lymphoproliferative disease. In another particular embodiment, the virus is human cytomegalovirus, Epstein-Barr virus, hepatitis virus, herpes virus, human rhinovirus, infectious laryngotracheitis virus, poliomyelitis virus, or varicella zoster virus. In a further particular embodiment, the parasite is 20 *Plasmodium falciparum*.

In a further aspect, the invention provides a pharmaceutical composition for treating a fungal infection, such as *Candida*, in a mammal comprising the recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

25 In yet another aspect, the invention provides a method of inhibiting or destroying cells affected by a disease, which cells are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease, comprising the steps of preparing a recombinant protein of the invention 30 having a heterologous linker sequence which contains a cleavage recognition site for the disease-specific protease and administering the recombinant protein to the cells. In an embodiment, the cancer is T-cell or B-cell lymphoproliferative disease, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast

5 zoster virus. In another embodiment, the parasite is *Plasmodium falciparum*.

10 an effective amount of one or more recombinant proteins of the invention
to said mammal.

15 cancer or infection with a virus, fungus, or a parasite each of which has a specific protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker
20 sequence contains a cleavage recognition site for the disease-specific protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker
25 sequence contains the cleavage recognition site for the disease-specific protease; and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

30 by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite each of which has a specific protease comprising the steps of identifying a cleavage recognition site for the protease; preparing a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous

linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

5 In a further aspect, the invention provides a pharmaceutical composition for treating for treating a mammal with disease wherein cells affected by the disease are associated with a disease specific protease, including cancer or infection with a virus, fungus, or a parasite comprising the recombinant protein of the invention and a pharmaceutically
10 acceptable carrier, diluent or excipient.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are
15 given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to
20 the drawings in which:

Figure 1 shows the DNA sequence of the baculovirus transfer vector, pVL1393;

Figure 2A summarizes the cloning strategy used to generate the pAP-213 construct;

25 Figure 2B shows the nucleotide sequence of the Cathepsin B linker regions of pAP-213;

Figure 2C shows the subcloning of the Cathepsin B linker variant into a baculovirus transfer vector;

30 Figure 2D shows the DNA sequence of the pAP-214 insert containing ricin and the Cathepsin B linker;

Figure 3A summarizes the cloning strategy used to generate the pAP-215 construct;

Figure 3B shows the nucleotide sequence of the MMP-3 linker regions of pAP-215;

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Figure 3C shows the subcloning of the MMP-3 linker variant into a baculovirus transfer vector;

Figure 3D shows the DNA sequence of the pAP-216 insert containing ricin and the MMP-3 linker;

5 Figure 4A summarizes the cloning strategy used to generate the pAP-217 construct;

Figure 4B shows the nucleotide sequence of the MMP-7 linker regions of pAP-217;

10 Figure 4C shows the subcloning of the MMP-7 linker variant into a baculovirus transfer vector;

Figure 4D shows the DNA sequence of the pAP-218 insert containing ricin and the MMP-7 linker;

Figure 5A summarizes the cloning strategy used to generate the pAP-219 construct;

15 Figure 5B shows the nucleotide sequence of the MMP-9 linker regions of pAP-219;

Figure 5C shows the subcloning of the MMP-9 linker variant into a baculovirus transfer vector;

20 Figure 5D shows the DNA sequence of the pAP-220 insert containing ricin and the MMP-9 linker.

Figure 6A summarizes the cloning strategy used to generate the pAP-221 construct;

Figure 6B shows the nucleotide sequence of the thermolysin-like MMP linker regions of pAP-221;

25 Figure 6C shows the subcloning of the thermolysin-like MMP linker variant into a baculovirus transfer vector.

Figure 6D shows the DNA sequence of the pAP-222 insert containing ricin and the thermolysin-like MMP linker;

30 Figure 7A summarizes the cloning strategy used to generate the pAP-223 construct;

Figure 7B shows the nucleotide sequence of the Plasmodium falciparum-A linker regions of pAP-223;

Figure 7C shows the subcloning of the Plasmodium falciparum-A linker variant into a baculovirus transfer vector;

Figure 7D shows the DNA sequence of the pAP-224 insert containing ricin and the Plasmodium falciparum-A linker;

Figure 8A summarizes the cloning strategy used to generate the pAP-225 construct;

5 Figure 8B shows the nucleotide sequence of the Plasmodium falciparum-B linker regions of pAP-225;

Figure 8C shows the subcloning of the Plasmodium falciparum-B linker variant into a baculovirus transfer vector;

10 Figure 8D shows the DNA sequence of the pAP-226 insert containing ricin and the Plasmodium falciparum-B linker;

Figure 9A summarizes the cloning strategy used to generate the pAP-227 construct;

Figure 9B shows the nucleotide sequence of the Plasmodium falciparum-C linker regions of pAP-227;

15 Figure 9C shows the subcloning of the Plasmodium falciparum-C linker variant into a baculovirus transfer vector;

Figure 9D shows the DNA sequence of the pAP-228 insert containing ricin and the Plasmodium falciparum-C linker;

20 Figure 10A summarizes the cloning strategy used to generate the pAP-229 construct;

Figure 10B shows the nucleotide sequence of the Plasmodium falciparum-D linker regions of pAP-229;

Figure 10C shows the subcloning of the Plasmodium falciparum-D linker variant into a baculovirus transfer vector;

25 Figure 10D shows the DNA sequence of the pAP-230 insert containing ricin and the Plasmodium falciparum-D linker;

Figure 11A summarizes the cloning strategy used to generate the pAP-231 construct;

30 Figure 11B shows the nucleotide sequence of the Plasmodium falciparum-E linker regions of pAP-231;

Figure 11C shows the subcloning of the Plasmodium falciparum-E linker variant into a baculovirus transfer vector;

Figure 11D shows the DNA sequence of the pAP-232 insert containing ricin and the Plasmodium falciparum-E linker;

Figure 12A summarizes the cloning strategy used to generate the pAP-233 construct;

Figure 12B shows the nucleotide sequence of the HSV-A linker regions of pAP-233;

5 Figure 12C shows the subcloning of the HSV-A linker variant into a baculovirus transfer vector;

Figure 12D shows the DNA sequence of the pAP-234 insert containing ricin and the HSV-A linker;

10 Figure 13A summarizes the cloning strategy used to generate the pAP-235 construct;

Figure 13B shows the nucleotide sequence of the HSV-B linker regions of pAP-235;

Figure 13C shows the subcloning of the HSV-B linker variant into a baculovirus transfer vector;

15 Figure 13D shows the DNA sequence of the pAP-236 insert containing ricin and the HSV-B linker;

Figure 14A summarizes the cloning strategy used to generate the pAP-237 construct;

20 Figure 14B shows the nucleotide sequence of the VZV-A linker regions of pAP-237;

Figure 14C shows the subcloning of the VZV-A linker variant into a baculovirus transfer vector;

Figure 14D shows the DNA sequence of the pAP-238 insert containing ricin and the VZV-A linker;

25 Figure 15A summarizes the cloning strategy used to generate the pAP-239 construct;

Figure 15B shows the nucleotide sequence of the VZV-B linker regions of pAP-239;

30 Figure 15C shows the subcloning of the VZV-B linker variant into a baculovirus transfer vector;

Figure 15D shows the DNA sequence of the pAP-240 insert containing ricin and the VZV-B linker;

Figure 16A summarizes the cloning strategy used to generate the pAP-241 construct;

Figure 16B shows the nucleotide sequence of the EBV-A linker regions of pAP-241;

Figure 16C shows the subcloning of the EBV-A linker variant into a baculovirus transfer vector;

5 Figure 16D shows the DNA sequence of the pAP-242 insert containing ricin and the EBV-A linker;

Figure 17A summarizes the cloning strategy used to generate the pAP-243 construct;

10 Figure 17B shows the nucleotide sequence of the EBV-B linker regions of pAP-243;

Figure 17C shows the subcloning of the EBV-B linker variant into a baculovirus transfer vector;

Figure 17D shows the DNA sequence of the pAP-244 insert containing ricin and the EBV-B linker;

15 Figure 18A summarizes the cloning strategy used to generate the pAP-245 construct;

Figure 18B shows the nucleotide sequence of the CMV-A linker regions of pAP-245;

20 Figure 18C shows the subcloning of the CMV-A linker variant into a baculovirus transfer vector;

Figure 18D shows the DNA sequence of the pAP-246 insert containing ricin and the CMV-A linker;

Figure 19A summarizes the cloning strategy used to generate the pAP-247 construct;

25 Figure 19B shows the nucleotide sequence of the CMV-B linker regions of pAP-247;

Figure 19C shows the subcloning of the CMV-B linker variant into a baculovirus transfer vector;

30 Figure 19D shows the DNA sequence of the pAP-248 insert containing ricin and the CMV-B linker.

Figure 20A summarizes the cloning strategy used to generate the pAP-249 construct;

Figure 20B shows the nucleotide sequence of the HHV-6 linker regions of pAP-249;

Figure 20C shows the subcloning of the HHV-6 linker variant into a baculovirus transfer vector;

Figure 20D shows the DNA sequence of the pAP-250 insert containing ricin and the HHV-6 linker;

5 Figure 21 shows the amino acid sequences of the wild type ricin linker and cancer protease-sensitive amino acid linkers contained in pAP-213 to pAP-222 and linkers pAP-241 to pAP-244;

Figure 22A summarizes the cloning strategy used to generate the pAP-253 construct;

10 Figure 22B shows the nucleotide sequence of the ILV linker regions of pAP-253;

Figure 22C shows the subcloning of the ILV linker variant into a baculovirus transfer vector;

15 Figure 22D shows the DNA sequence of the pAP-254 insert containing ricin and the ILV linker;

Figure 23A summarizes the cloning strategy used to generate the pAP-257 construct;

Figure 23B shows the nucleotide sequence of the HAV-A linker regions of pAP-257;

20 Figure 23C shows the subcloning of the HAV-A linker variant into a baculovirus transfer vector;

Figure 23D shows the DNA sequence of the pAP-258 insert containing ricin and the HAV-A linker;

25 Figure 24A summarizes the cloning strategy used to generate the pAP-255 construct;

Figure 24B shows the nucleotide sequence of the HAV-B linker regions of pAP-255;

Figure 24C shows the subcloning of the HAV-B linker variant into a baculovirus transfer vector;

30 Figure 24D shows the DNA sequence of the pAP-256 insert containing ricin and the HAV-B linker;

Figure 25A summarizes the cloning strategy used to generate the pAP-259 construct;

095151-04400

Figure 25B shows the nucleotide sequence of the CAN linker regions of pAP-259;

Figure 25C shows the subcloning of the CAN linker variant into a baculovirus transfer vector;

5 Figure 25D shows the DNA sequence of the pAP-260 insert containing ricin and the CAN linker;

Figure 26 shows the amino acid sequences of the wild type ricin linker and *Plasmodium falciparum* protease-sensitive amino acid linkers contained in linkers pAP-223 to pAP-232;

10 Figure 27 shows the amino acid sequences of the wild type ricin linker and the viral protease-sensitive amino acid linkers contained in pAP-233 to pAP-240, pAP-245-pAP-248, pAP-253 to pAP-258;

Figure 28 shows the amino acid sequences of the wild type ricin linker and the *Candida* aspartic protease-sensitive amino acid linker
15 contained in pAP-259 to pAP-264;

Figure 29 describes an alternative mutagenesis and subcloning strategy to provide a baculovirus transfer vector containing the ricin-like toxin variant gene; and

Figure 30A summarizes the cloning strategy used to
20 generate the pAP-262 construct;

Figure 30B shows the nucleotide sequence of the HCV-A linker region of pAP-262;

Figure 30C shows the DNA sequence of the pAP-262 insert;

Figure 30D shows the amino acid sequence comparison of
25 mutant preproricin linker region HCV-A to wild type;

Figure 31A summarizes the cloning strategy used to generate the pAP-264 construct;

Figure 31B shows the nucleotide sequence of the HCV-B linker region of pAP-264;

30 Figure 31C shows the DNA sequence of the pAP-264 insert;

Figure 31D shows the amino acid sequence comparison of mutant preproricin linker region HCV-B to wild type;

Figure 32A summarizes the cloning strategy used to generate the pAP-266 construct;

Figure 32B shows the nucleotide sequence of the HCV-C linker region of pAP-266;

Figure 32C shows the DNA sequence of the pAP-266 insert;

Figure 32D shows the amino acid sequence comparison of
5 mutant preproricin linker region HCV-C to wild type;

Figure 33A summarizes the cloning strategy used to generate the pAP-268 construct;

Figure 33B shows the nucleotide sequence of the HCV-D linker region of pAP-268;

10 Figure 33C shows the DNA sequence of the pAP-268 insert;

Figure 33D shows the amino acid sequence comparison of mutant preproricin linker region HCV-D to wild type;

Figure 34A summarizes the cloning strategy used to generate the pAP-270 construct;

15 Figure 34B shows the nucleotide sequence of the MMP-2 linker region of pAP-270;

Figure 34C shows the DNA sequence of the pAP-270 insert;

Figure 34D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-2 to wild type;

20 Figure 35A summarizes the cloning strategy used to generate the pAP-272 construct;

Figure 35B shows the nucleotide sequence of the Cathepsin B (Site 2) linker region of pAP-272;

Figure 35C shows the DNA sequence of the pAP-272 insert;

25 Figure 35D shows the amino acid sequence comparison of mutant preproricin linker region of Cathepsin B (Site 2) to wild type;

Figure 36A summarizes the cloning strategy used to generate the pAP-274 construct;

30 Figure 36B shows the nucleotide sequence of the Cathepsin L linker region of pAP-274;

Figure 36C shows the DNA sequence of the pAP-274 insert;

Figure 36D shows the amino acid sequence comparison of mutant preproricin linker region of Cathepsin L to wild type;

Figure 37A summarizes the cloning strategy used to generate the pAP-276 construct;

Figure 37B shows the nucleotide sequence of the Cathepsin D linker region of pAP-276;

5 Figure 37C shows the DNA sequence of the pAP-276 insert;

Figure 37D shows the amino acid sequence comparison of mutant preproricin linker region of Cathepsin D to wild type;

Figure 38A summarizes the cloning strategy used to generate the pAP-278 construct;

10 Figure 38B shows the nucleotide sequence of the MMP-1 linker region of pAP-278;

Figure 38C shows the DNA sequence of the pAP-278 insert;

Figure 38D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-1 to wild type;

15 Figure 39A summarizes the cloning strategy used to generate the pAP-280 construct;

Figure 39B shows the nucleotide sequence of the Urokinase-Type Plasminogen Activator linker region of pAP-280;

Figure 39C shows the DNA sequence of the pAP-280 insert;

20 Figure 39D shows the amino acid sequence comparison of mutant preproricin linker region of Urokinase-Type Plasminogen Activator to wild type;

Figure 40A summarizes the cloning strategy used to generate the pAP-282 construct;

25 Figure 40B shows the nucleotide sequence of the MT-MMP linker region of pAP-282;

Figure 40C shows the DNA sequence of the pAP-282 insert;

Figure 40D shows the amino acid sequence comparison of mutant preproricin linker region of MT-MMP to wild type;

30 Figure 41A summarizes the cloning strategy used to generate the pAP-284 construct;

Figure 41B shows the nucleotide sequence of the MMP-11 linker region of pAP-284;

Figure 41C shows the DNA sequence of the pAP-284 insert;

Figure 41D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-11 to wild type;

Figure 42A summarizes the cloning strategy used to generate the pAP-286 construct;

5 Figure 42B shows the nucleotide sequence of the MMP-13 linker region of pAP-286;

Figure 42C shows the DNA sequence of the pAP-286 insert;

Figure 42D shows the amino acid sequence comparison of mutant preproricin linker region of MMP-13 to wild type;

Figure 43A summarizes the cloning strategy used to generate the pAP-288 construct;

Figure 43B shows the nucleotide sequence of the Tissue-type Plasminogen Activator linker region of pAP-288;

Figure 43C shows the DNA sequence of the pAP-288 insert;

15 Figure 43D shows the amino acid sequence comparison of mutant preproricin linker region of Tissue-type Plasminogen Activator to wild type;

Figure 44A summarizes the cloning strategy used to generate the pAP-290 construct;

20 Figure 44B shows the nucleotide sequence of the human Prostate-Specific Antigen linker region of pAP-290;

Figure 44C shows the DNA sequence of the pAP-290 insert;

Figure 44D shows the amino acid sequence comparison of mutant preproricin linker region of the human Prostate-Specific Antigen to wild type;

Figure 45A summarizes the cloning strategy used to generate the pAP-292 construct;

Figure 45B shows the nucleotide sequence of the kallikrein linker region of pAP-292;

30 Figure 45C shows the DNA sequence of the pAP-292 insert;

Figure 45D shows the amino acid sequence comparison of mutant preproricin linker region of the kallikrein to wild type;

Figure 46A summarizes the cloning strategy used to generate the pAP-294 construct;

Figure 46B shows the nucleotide sequence of the neutrophil elastase linker region of pAP-294;

Figure 46C shows the DNA sequence of the pAP-294 insert;

Figure 46D shows the amino acid sequence comparison of
5 mutant preproricin linker region of neutrophil elastase to wild type;

Figure 47A summarizes the cloning strategy used to generate the pAP-296 construct;

Figure 47B shows the nucleotide sequence of the calpain linker region of pAP-296;

10 Figure 47C shows the DNA sequence of the pAP-296 insert;

Figure 47D shows the amino acid sequence comparison of mutant preproricin linker region of calpain to wild type;

Figure 48 is a blot showing cleavage of pAP-214 by
Cathepsin B;

15 Figure 49 is a blot showing cleavage of pAP-220 with
MMP-9;

Figure 50 is a blot showing activation of pAP-214; and

Figure 51 is a blot showing activation of pAP-220.

Figure 52 is a blot showing cleavage of pAP-248 with
20 HCMV.

Figure 53 is a blot showing activation of pAP-248.

Figure 54 is a blot showing cleavage of pAP-256 by HAV
3C.

Figure 55 is a blot showing activation of pAP-256.

25 Figure 56 is a semi-logithmic graph illustrating the
cytotoxicity to COS-1 cells of undigested pAP-214 and pAP-214
digested with Cathepsin B.

Figure 57 is a semi-logithmic graph illustrating the
cytotoxicity of pAP-220 digested with MMP-9 compared to freshly
30 thawed pAP-220 and ricin on COS-1 cells.

Figure 58 is a blot showing cleavage of pAP-270 with
MMP-2.

Figure 59 is a blot showing activation of pAP-270.

Figure 60 is a blot showing cleavage of pAP-288 by t-PA.

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Figure 61 is a blot showing activation of pAP-288.

Figure 62 is a blot showing cleavage of pAP-294 with human neutrophil elastase.

Figure 63 is a blot showing activation of pAP-294.

5 Figure 64 is a blot showing cleavage of pAP-296 with calpain.

Figure 65 is a blot showing activation of pAP-296.

Figure 66 is a blot showing cleavage of pAP-222 with MMP-2.

10 Figure 67 is a blot showing activation of pAP-222.

DETAILED DESCRIPTION OF THE INVENTION

Nucleic Acid Molecules of the Invention

As mentioned above, the present invention relates to novel nucleic acid molecules comprising a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The heterologous linker sequence contains a cleavage recognition site for a disease-specific protease (e.g. a viral protease, parasitic protease, cancer-associated protease, or a fungal protease).

20 The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of sequences which naturally flank the nucleic acid (*i.e.* sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

30 The term "linker sequence" as used herein refers to an internal amino acid sequence within the protein encoded by the nucleic acid molecule of the invention which contains residues linking the A and B chain so as to render the A chain incapable of exerting its toxic effect, for example catalytically inhibiting translation of a eukaryotic ribosome. By heterologous is meant that the linker sequence is not a sequence native to

the A or B chain of a ricin-like toxin or precursor thereof. However, preferably, the linker sequence may be of a similar length to the linker sequence of a ricin-like toxin and should not interfere with the role of the B chain in cell binding and transport into the cytoplasm. When the linker
5 sequence is cleaved the A chain becomes active or toxic.

The nucleic acid molecule of the invention is cloned by subjecting a preproricin cDNA clone to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). Oligonucleotides, corresponding to the
10 extreme 5' and 3' ends of the preproricin gene are synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.* 145:266-270 (1985)), several oligonucleotide primers are designed to flank the start and stop codons of the preproricin open reading frame.

The preproricin cDNA is amplified using the upstream primer Ricin-99 or Ricin-109 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). The purified PCR
20 fragment encoding the preproricin cDNA is then ligated into an Eco RI-digested pBluescript II SK plasmid (Stratagene), and is used to transform competent XL1-Blue cells (Stratagene). The cloned PCR product containing the putative preproricin gene is confirmed by DNA sequencing of the entire cDNA clone. The sequences and location of oligonucleotide
25 primers used for sequencing are shown in Table 1.

The preproricin cDNA clone is subjected to site directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region is replaced with the heterogenous linker
30 sequences that are cleaved by the various disease-specific proteases as shown in Figures 21, 26, 27, 28, and Part D of Figures 30-47. Linker identification as used herein in connection with the sequences provided in these figures have been assigned the sequence ID numbers as discussed below.

The linker regions of the variants encode a cleavage recognition sequence for a disease-specific protease associated with for example, cancer, viruses, parasites, or fungi. The mutagenesis and cloning strategy used to generate the disease-specific protease-sensitive linker variants are summarized in Part A of Figures 2-20, and Part A of Figures 22-25. The first step involves a DNA amplification using a set of mutagenic primers in combination with the two flanking primers Richin-99Eco or Ricin-109Eco and Ricin1729C Pst I. Restriction digested PCR fragments are gel purified and then ligated with PBluescript SK which has been digested with Eco RI and Pst I. Ligation reactions are used to transform competent XL1-Blue cells (Stratagene). Recombinant clones are identified by restriction digests of plasmid miniprep DNA and the mutant linker sequences are confirmed by DNA sequencing. With respect to the nucleotide sequences and amino acid sequences prepared as a result of the implementation of this strategy the following sequences have been assigned the sequence ID numbers as indicated.

SEQ ID NO. 1 is used herein in connection with the DNA sequence of the baculovirus transfer vector, pVL1393.

The nucleotide sequence of Cathepsin B linker regions of pAP-213 are referred to herein as SEQ ID NO. 2.

The nucleotide sequence of Cathepsin B linker regions of pAP-214 are referred to herein as SEQ ID NO. 3.

The nucleotide sequence of MMP-3 linker regions of pAP-215 are referred to herein as SEQ ID NO. 4.

The DNA sequence of the pAP-216 insert containing ricin and the MMP-3 linker are referred to herein as SEQ ID NO. 5.

The nucleotide sequence of MMP-7 linker regions of pAP-217 are referred to herein as SEQ ID NO. 6.

The DNA sequence of the pAP-218 insert containing ricin and the MMP-7 linker are referred to herein as SEQ ID NO. 7.

The nucleotide sequence of MMP-9 linker regions of pAP-219 are referred to herein as SEQ ID NO. 8.

The DNA sequence of the pAP-220 insert containing ricin and the MMP-9 are referred to herein as SEQ ID NO. 9.

The nucleotide sequence of thermolysin-like MMP linker regions of pAP-221 are referred to herein as SEQ ID NO. 10.

The DNA sequence of of pAP-222 insert containing ricin and the thermolysin-like MMP linker are referred to herein as SEQ ID NO. 11.

5 The nucleotide sequence of Plasmodium falciparum-A linker regions of pAP-223 are referred to herein as SEQ ID NO. 12.

The DNA sequence of the pAP-224 insert containing ricin and the Plasmodium falciparum-A linker are referred to herein as SEQ ID NO. 13.

10 The nucleotide sequence of Plasmodium falciparum-B linker regions of pAP-225 are referred to herein as SEQ ID NO. 14.

The DNA sequence of the pAP-226 insert containing ricin and the Plasmodium falciparum-B linker are referred to herein as SEQ ID NO. 15.

15 The nucleotide sequence of Plasmodium falciparum-C linker regions of pAP-227 are referred to herein as SEQ ID NO. 16.

The DNA sequence of the pAP-228 insert containing ricin and the Plasmodium falciparum-C linker are referred to herein as SEQ ID NO. 17.

20 The nucleotide sequence of the the Plasmodium falciparum-D linker regions of pAP-229 is referred to herein as SEQ ID NO. 18.

The DNA sequence of the pAP-230 insert containing ricin and the Plasmodium falciparum-D linker is referred to herein as SEQ ID NO. 19.

25 The nucleotide sequence of the Plasmodium falciparum-E linker regions of pAP-231 is referred to herein as SEQ ID NO. 20.

The DNA sequence of the pAP-232 insert containing ricin and the Plasmodium falciparum-E linker is referred to herein as SEQ ID NO. 21.

30 The nucleotide sequence of the HSV-A linker regions of pAP-233 is referred to herein as SEQ ID NO. 22.

The DNA sequence of the pAP-234 insert containing ricin and the HSV-A linker is referred to herein as SEQ ID NO. 23.

The nucleotide sequence of the HSV-B linker regions of pAP-235 is referred to herein as SEQ ID NO. 24.

The DNA sequence of the pAP-236 insert containing ricin and the HSV-B linker is referred to herein as SEQ ID NO. 25.

5 The nucleotide sequence of the VZV-A linker regions of pAP-237 are referred to herein as SEQ ID NO. 26.

The DNA sequence of the pAP-238 insert containing ricin and the VZV-A linker are referred to herein as SEQ ID NO. 27.

10 The nucleotide sequence of the VZV-B linker regions of PAP-239 is referred to herein as SEQ ID NO. 28.

The DNA sequence of the pAP-240 insert containing ricin and the VZV-B linker is referred to herein as SEQ ID NO. 29.

The nucleotide sequence of the EBV-A linker regions of pAP-241 is referred to herein as SEQ ID NO. 30.

15 The DNA sequence of the pAP-242 insert containing ricin and the EBV-A linker is referred to herein as SEQ ID NO. 31.

The nucleotide sequence of the EBV-B linker regions of pAP-243 is referred to herein as SEQ ID NO. 32.

20 The DNA sequence of the pAP-244 insert containing ricin and the EBV-B linker is referred to herein as SEQ ID NO. 33.

The nucleotide sequence of the CMV-A linker regions of pAP-245 is referred to herein as SEQ ID NO. 34.

The DNA sequence of the pAP-246 insert containing ricin and the CMV-A linker is referred to herein as SEQ ID NO. 35.

25 The nucleotide sequence of the CMV-B linker regions of pAP-247 is referred to herein as SEQ ID NO. 36.

The DNA sequence of the pAP-248 insert containing ricin and the CMV-B linker is referred to herein as SEQ ID NO. 37.

30 The nucleotide sequence of the HHV-6 linker regions of pAP-249 is referred to herein as SEQ ID NO. 38.

The DNA sequence of the pAP-250 insert containing ricin and the HHV-6 linker is referred to herein as SEQ ID NO. 39.

The amino acid sequences of the cancer protease-sensitive amino acid linkers contained in the following pAP proteins have the

sequence ID numbers as indicated: pAP-213 and pAP-214 (SEQ ID NO. 40); pAP-215 and pAP-216 (SEQ ID NO. 41); pAP-217 and pAP-218; (SEQ ID NO. 42); pAP-219 and pAP-220 (SEQ ID NO. 43); and pAP-221 and pAP-222 (SEQ ID NO. 44).

5 The amino acid sequences of the following cancer protease-sensitive linkers are referred to herein with the corresponding sequence ID numbers: pAP-241 and pAP-242 (SEQ ID NO. 45); and pAP-243 and pAP-244 (SEQ ID NO. 46).

10 The nucleotide sequence of the ILV linker regions of pAP-253 is referred to herein as SEQ ID NO. 47.

 The DNA sequence of the pAP-254 insert containing ricin and the ILV linker is referred to herein as SEQ ID NO. 48.

 The nucleotide sequence of the HAV-A linker regions of pAP-257 is referred to herein as SEQ ID NO. 49.

15 The DNA sequence of the pAP-258 insert containing ricin and HAV-A linker is referred to herein as SEQ ID NO. 50.

 The nucleotide sequence of the HAV-B linker regions of pAP-255 is referred to herein as SEQ ID NO. 51.

20 The DNA sequence of the pAP-256 insert containing ricin and the HAV-B linker is referred to herein as SEQ ID NO. 52.

 The nucleotide sequence of the CAN linker regions of pAP-259 is referred to herein as SEQ ID NO. 53.

 The DNA sequence of the pAP-260 insert containing ricin and the CAN linker is referred to herein as SEQ ID NO. 54.

25 The amino acid sequences of Plasmodium falciparum protease-sensitive linkers are referred to herein by the sequence ID numbers as follows: pAP-223 and pAP-224 (SEQ ID NO 55); pAP-225 and pAP-226 (SEQ ID NO 56); pAP-227 and pAP-228 (SEQ ID NO 57); pAP-229 and pAP-230 (SEQ ID NO 58); and pAP-231 and pAP-232 (SEQ ID NO 59)
30 (see Figure 26).

 The amino acid sequences of the viral protease-sensitive linkers which follow are referred to herein by the sequence ID numbers indicated: pAP-233 and pAP 234 (SEQ ID NO 60); pAP-235 and pAP-236 (SEQ ID NO 61); and pAP-249 and pAP-250 (SEQ ID NO 62) (see Figure 27).

The amino acid sequences of the viral protease-sensitive linkers which follow are referred to herein by the sequence ID numbers indicated: pAP-245 and pAP-246 (SEQ ID NO 63) ; and pAP-247 and pAP-248 (SEQ ID NO 64) (see Figure 27).

5 The amino acid sequences of the viral protease-sensitive linkers which follow are referred to herein by the sequence ID numbers indicated: pAP-237 and pAP-238 (SEQ ID NO 65); and pAP-239 and pAP-240 (SEQ ID NO 66); pAP-253 and pAP-254 (SEQ ID NO 67); pAP-255 and pAP-256 (SEQ ID NO 68); and pAP-257 and pAP-258 (SEQ ID NO 69) (see
10 Figure 27).

The amino acid sequences of the *Candida* aspartic protease-sensitive linkers are referred to herein by the sequence ID numbers indicated: pAP-259 and pAP-260 (SEQ ID NO 70); pAP-261 and pAP-262 (SEQ ID NO 71); and pAP-263 and pAP-264 (SEQ ID NO 72).

15 An alternative mutagenesis and cloning strategy that can be used to generate the disease-specific protease-sensitive linker variants is summarized in Figure 29. The first step of this method involves a DNA amplification using a set of mutagenic primers in combination with the two flanking primers Ricin-109Eco and Ricin1729Pst. Restriction digested PCR
20 fragments (Eco RI and Pst I) are gel purified. Preproricin variants produced from this method can be subcloned directly into the baculovirus transfer vector digested with Eco RI and Pst I and intermediate ligation steps involving pBluescript SK and pSB2 are circumvented. The cloning strategies used to generate disease-specific protease-sensitive linker
25 variants are summarized in Part A of Figures 30 to 47. With respect to the nucleotide sequences and amino acid sequences prepared as a result of the implementation of this strategy the following sequences have been assigned the sequence ID numbers as indicated.

30 The nucleotide sequence of the HCV-A linker region of pAP-262 is referred to herein as SEQ ID NO. 73.

The DNA sequence of the pAP-262 insert is referred to herein as SEQ ID NO. 74.

The amino acid sequence of the mutant preproricin linker region for HCV-A, pAP-262, is referred to herein as SEQ ID NO. 75.

The nucleotide sequence of the HCV-B linker region of pAP-264 is referred to herein as SEQ ID NO. 76.

The DNA sequence of the pAP-264 insert is referred to herein as SEQ ID NO. 77.

5 The amino acid sequence of the mutant preproricin linker region for HCV-B, pAP-264, is referred to herein as SEQ ID NO. 78.

The nucleotide sequence of the HCV-C linker region of pAP-266 is referred to herein as SEQ ID NO. 79.

10 The DNA sequence of the pAP-266 insert is referred to herein as SEQ ID NO. 80.

The amino acid sequence of the mutant preproricin linker region for HCV-C, pAP-266, is referred to herein as SEQ ID NO. 81.

The nucleotide sequence of the HCV-D linker region of pAP-268 is referred to herein as SEQ ID NO. 82.

15 The DNA sequence of the pAP-268 insert is referred to herein as SEQ ID NO. 83.

The amino acid sequence of the mutant preproricin linker region for HCV-D , pAP-268, is referred to herein as SEQ ID NO. 84.

20 The nucleotide sequence of the MMP-2 linker region of pAP-270 is referred to herein as SEQ ID NO. 85.

The DNA sequence of the pAP-270 insert is referred to herein as SEQ ID NO. 86.

The amino acid sequence of the mutant preproricin linker region for MMP-2, pAP-270, is referred to herein as SEQ ID NO. 87.

25 The nucleotide acid sequence of the Cathepsin B (Site 2) linker region of pAP-272 is referred to herein as SEQ ID NO. 88.

The DNA sequence of the pAP-272 insert is referred to herein as SEQ ID NO. 89.

30 The amino acid sequence of the mutant preproricin linker region for Cathepsin B (Site 2), pAP-272, is referred to herein as SEQ ID NO. 90.

The nucleotide sequence of the Cathepsin L linker region of pAP-274 is referred to herein as SEQ ID NO. 91.

The DNA sequence of the pAP-274 insert is referred to herein as SEQ ID NO. 92.

The amino acid sequence of the mutant preprorin linker region of Cathepsin L, pAP-274, is referred to herein as SEQ ID NO. 93.

5 The nucleotide sequence of Cathepsin D linker region of pAP-276 is referred to herein as SEQ ID NO. 94.

The DNA sequence of the pAP-276 insert is referred to herein as SEQ ID NO. 95.

10 The amino acid sequence of the mutant preprorin linker region for Cathepsin D, pAP-276, is referred to herein as SEQ ID NO. 96.

The nucleotide sequence of the MMP-1 linker region of pAP-278 is referred to herein as SEQ ID NO. 97.

The DNA sequence of the pAP-278 insert is referred to herein as SEQ ID NO. 98.

15 The amino acid sequence of the mutant preprorin linker region for MMP-1, pAP-278, is referred to herein as SEQ ID NO. 99.

The nucleotide sequence of the Urokinase-Type Plasminogen Activator linker region of pAP-280 is referred to herein as SEQ ID NO. 100.

20 The DNA sequence of the pAP-280 insert is referred to herein as SEQ ID NO. 101.

The amino acid sequence of the mutant preprorin linker region for Urokinase-Type Plasminogen Activator, pAP-280, is referred to herein as SEQ ID NO. 102.

25 The nucleotide sequence of MT-MMP linker region of pAP-282 is referred to herein as SEQ ID NO. 103.

The DNA sequence of the pAP-282 insert is referred to herein as SEQ ID NO. 104.

30 The amino acid sequence of the mutant preprorin linker region for MT-MMP, pAP-282, is referred to herein as SEQ ID NO. 105.

The nucleotide sequence of the MMP-11 linker region of pAP-284 is referred to herein as SEQ ID NO. 106.

The DNA sequence of the pAP-284 insert is referred to herein as SEQ ID NO. 107.

The amino acid sequence of the mutant preproricin linker region for neutrophil elastase, pAP-294, is referred to herein as SEQ ID NO. 123.

5 The nucleotide sequence of the calpain linker region of pAP-296 is referred to herein as SEQ ID NO. 124.

The DNA sequence of the pAP-296 insert is referred to herein as SEQ ID NO. 125.

The amino acid sequence of the mutant preproricin linker region for calpain, pAP-296, is referred to herein as SEQ ID NO. 126.

10 The amino acid sequence of the wild type linker region is referred to herein as SEQ ID NO. 127.

The nucleic acid molecule of the invention has sequences encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease. The nucleic acid may be expressed to provide a recombinant protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease.

20 The nucleic acid molecule may comprise the A and/or B chain of ricin. The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains are published (Rutenber, E., et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Biol.* 244:410-422 (1994); Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K., et al., *Nucleic Acids Res.* 13:8019 (1985)). It will be appreciated that the invention includes nucleic acid molecules encoding truncations of A and B chains of ricin like proteins and analogs and homologs of A and B chains of ricin-like proteins and truncations thereof (i.e., ricin-like proteins), as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention.

30 Another aspect of the invention provides a nucleotide sequence which hybridizes under high stringency conditions to a nucleotide sequence encoding the A and/or B chains of a ricin-like protein.

Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1 6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

The nucleic acid molecule may comprise the A and/or B chain of a ricin-like toxin. Methods for cloning ricin-like toxins are known in the art and are described, for example, in E.P. 466,222. Sequences encoding ricin or ricin-like A and B chains may be obtained by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from the nucleic acid sequence of A and B chains of ricin or ricin-like toxins. It is also possible to design synthetic oligonucleotide primers from the nucleotide sequences for use in PCR. Suitable primers may be selected from the sequences encoding regions of ricin-like proteins which are highly conserved, as described for example in U.S. Patent No 5,101,025 and E.P. 466,222.

A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., *Biochemistry* 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL). It will be appreciated that the methods described above may be used to obtain the coding sequence from plants, bacteria or fungi, preferably

plants, which produce known ricin-like proteins and also to screen for the presence of genes encoding as yet unknown ricin-like proteins.

5 A sequence containing a cleavage recognition site for a specific protease may be selected based on the disease or the pathogen which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the cancer, viral or parasitic protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to cleavage by the
10 respective protease.

A sequence containing a cleavage recognition site for a viral, fungal, parasitic or cancer associated protease may be selected based on the retrovirus which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to
15 encode a cleavage recognition site for the viral, fungal, parasitic or cancer associated protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to cleavage by a viral, fungal, parasitic or cancer associated protease. A polypeptide containing the suspected cleavage recognition site
20 may be incubated with a protease and the amount of cleavage product determined (DiIannit, 1990, J. Biol. Chem. 285: 17345-17354 (1990)).

The protease may be prepared by methods known in the art and used to test suspected cleavage recognition sites.

In one embodiment, the preparation of tumour-associated
25 cathepsin B, its substrates and enzymatic activity assay methodology have been described by Sloane, B.F. et al. (*Proc. Natl. Acad. Sci. USA* 83:2483-2487 (1986)), Schwartz, M.K. (*Clin. Chim. Acta* 237:67-78 (1995)), and Panchal, R.G. et al. (*Nature Biotechnol.* 14:852-856 (1996)). The preparation of Epstein-Barr virus protease, its substrates and enzymatic activity assay
30 methodology have been described by Welch, A.R. (*Proc. Natl. Acad. Sci. USA* 88:10792-10796 (1991)).

In another embodiment, the preparation of *Plasmodium falciparum* proteases, their substrates and enzymatic activity assay methodology have been described by Goldberg, D.E. et al. (*J. Exp. Med.*

173:961-969 (1991)), Cooper & Bujard (*Mol. Biochem. Parasitol.* 56:151-160 (1992)), Nwagwu, M. et al. (*Exp. Parasitol.* 75:399-414 (1992)), Rosenthal, P.J. et al. (*J. Clin. Invest.* 91:1052-1056 (1993)), Blackman, M.J. et al. (*Mol. Biochem. Parasitol.* 62:103-114 (1995)).

5 In a further embodiment, the preparation of proteases from human cytomegalovirus, human herpes virus, varicella zoster virus and infectious laryngotracheitis virus have been taught by Liu F. & Roizman, B. (*J. Virol.* 65:5149-5156 (1991)) and Welch, A.R. (*Proc. Natl. Acad. Sci. USA* 88:10792-10796 (1991)). In addition, their respective substrates and
10 enzymatic activity assay methodologies are also described.

 In another embodiment, the preparation of hepatitis A virus protease, its substrates and enzymatic activity assay methodology have been described by Jewell, D.A. et al. (*Biochemistry* 31:7862-7869 (1992)). The preparation of poliovirus protease, its substrates and enzymatic activity
15 assay methodology have been described by Weidner, J.R. et al. (*Arch. Biochem. Biophys.* 286:402-408 (1991)). The preparation of human rhinovirus protease, its substrates and enzymatic activity assay methodology have been described by Long, A.C. et al. (*FEBS Lett.* 258:75-78 (1989)).

 In another embodiment of the invention, the preparation of
20 proteases associated with *Candida* yeasts their substrates and enzymatic activity are contemplated, including the aspartic proteinases which have been associated specifically with numerous virulent strains of *Candida* including *Candida albican*, *Candida tropicalis*, and *Candida parapsilosis* (Abad-Zapatero, C. et al., *Protein Sci.* 5:640-652 (1996); Cutfield, S.M. et al.,
25 *Biochemistry* 35:398-410 (1995); Ruchel, R. et al, *Zentralbl. Bakteriол. Mikrobiol Hyg. I Abt. Orig. A.* 255:537-548 (1983); Remold, H. et al., *Biochim. Biophys. Acta* 167:399-406 (1968)).

 The nucleic acid molecule of the invention may be prepared by site directed mutagenesis. For example, the cleavage site of a disease-
30 specific protease may be prepared by site directed mutagenesis of the homologous linker sequence of a proricin-like toxin. Procedures for cloning proricin-like genes, encoding a linker sequence are described in EP 466,222. Site directed mutagenesis may be accomplished by DNA amplification of mutagenic primers in combination with flanking primers.

Suitable procedures using the mutagenic primers are shown in Parts A and B of Figures 1-4, Figures 13-16, Figures 18-36, Figures 38-41, and Figures 50-67.

5 The nucleic acid molecule of the invention may also encode
a fusion protein. A sequence encoding a heterologous linker sequence
containing a cleavage recognition site for a disease-specific protease may
be cloned from a cDNA or genomic library or chemically synthesized
based on the known sequence of such cleavage sites. The heterologous
linker sequence may then be fused in frame with the sequences encoding
10 the A and B chains of the ricin-like toxin for expression as a fusion protein.
It will be appreciated that a nucleic acid molecule encoding a fusion protein
may contain a sequence encoding an A chain and a B chain from the same
ricin-like toxin or the encoded A and B chains may be from different toxins.
For example, the A chain may be derived from ricin and the B chain may
15 be derived from abrin. A protein may also be prepared by chemical
conjugation of the A and B chains and linker sequence using conventional
coupling agents for covalent attachment.

An isolated and purified nucleic acid molecule of the
invention which is RNA can be isolated by cloning a cDNA encoding an A
20 and B chain and a linker into an appropriate vector which allows for
transcription of the cDNA to produce an RNA molecule which encodes a
protein of the invention. For example, a cDNA can be cloned downstream
of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can
be transcribed in vitro with T7 polymerase, and the resultant RNA can be
25 isolated by standard techniques.

Recombinant Protein of the Invention

As previously mentioned, the invention provides novel
recombinant proteins which incorporate the A and B chains of a ricin like
toxin linked by a heterologous linker sequence containing a cleavage
30 recognition site for a disease-specific protease. It is an advantage of the
recombinant proteins of the invention that they are non-toxic until the A
chain is liberated from the B chain by specific cleavage of the linker by the
target protease.

Thus the protein may be used to specifically target cancer cells or cells infected with a virus or parasite in the absence of additional specific cell-binding components to target infected cells. It is a further advantage that the disease-specific protease cleaves the heterologous linker intracellularly thereby releasing the toxic A chain directly into the cytoplasm of the cancer cell or infected cell. As a result, said cells are specifically targeted and non-infected normal cells are not directly exposed to the activated free A chain.

Ricin is a plant derived ribosome inhibiting protein which blocks protein synthesis in eukaryotic cells. Ricin may be derived from the seeds of *Ricinus communis* (castor oil plant). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. Biol. Chem. 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)).

All protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside plant cells. The A chain is inactive in the proricin (O'Hare, M., et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20

(1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell.

Ricin-like proteins include, but are not limited to, bacterial, 5 fungal and plant toxins which have A and B chains and inactivate ribosomes and inhibit protein synthesis. The A chain is an active polypeptide subunit which is responsible for the pharmacologic effect of the toxin. In most cases the active component of the A chain is an enzyme. The B chain is responsible for binding the toxin to the cell surface and is 10 thought to facilitate entry of the A chain into the cell cytoplasm. The A and B chains in the mature toxins are linked by disulfide bonds. The toxins most similar in structure to ricin are plant toxins which have one A chain and one B chain. Examples of such toxins include abrin which may be isolated from the seeds of *Abrus precatorius*, modeccin, volkensin and 15 viscumin.

Ricin-like bacterial proteins include diphtheria toxin, which is produced by *Corynebacterium diphtheriae*, *Pseudomonas enterotoxin A* and cholera toxin. It will be appreciated that the term ricin-like toxins is also intended to include the A chain of those toxins which have only an A 20 chain. The recombinant proteins of the invention could include the A chain of these toxins conjugated to, or expressed as, a recombinant protein with the B chain of another toxin. Examples of plant toxins having only an A chain include trichosanthin, MMC and pokeweed antiviral proteins, dianthin 30, dianthin 32, croton II, curcin II and wheat germ inhibitor. 25 Examples of fungal toxins having only an A chain include alpha-sarcin, restrictocin, mitogillin, enomycin, phenomycin. Examples of bacterial toxins having only an A chain include cytotoxin from *Shigella dysenteriae* and related Shiga-like toxins. Recombinant trichosanthin and the coding sequence thereof is disclosed in U.S. Patents 5,101,025 and 5,128,460.

30 In addition to the entire A or B chains of a ricin-like toxin, it will be appreciated that the recombinant protein of the invention may contain only that portion of the A chain which is necessary for exerting its cytotoxic effect. For example, the first 30 amino acids of the ricin A chain may be removed resulting in a truncated A chain which retains toxic

activity. The truncated ricin or ricin-like A chain may be prepared by expression of a truncated gene or by proteolytic degradation, for example with Nagarase (Funmatsu et al., *Jap. J. Med. Sci. Biol.* 23:264-267 (1970)). Similarly, the recombinant protein of the invention may contain only that

5 portion of the B chain necessary for galactose recognition, cell binding and transport into the cell cytoplasm. Truncated B chains are described for example in E.P. 145,111. The A and B chains may be glycosylated or non-glycosylated. Glycosylated A and B chains may be obtained by expression in the appropriate host cell capable of glycosylation.

10 Non-glycosylated chains may be obtained by expression in nonglycosylating host cells or by treatment to remove or destroy the carbohydrate moieties.

The proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the

15 present invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is

20 compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is

25 intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences

30 for the transcription and translation of the inserted protein-sequence.

Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic

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Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or
5 RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into
10 the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host
15 cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an
20 immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant
25 cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers
30 can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a

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ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Amrad
5 Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors can be introduced into host
10 cells to produce a transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass
15 introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium
20 chloride co-precipitation, DEAE-dextran mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

25 Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185,
30 Academic Press, San Diego, CA (1991).

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genus' *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one

of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β -lactamase (penicillinase) and

5 lactose promoter system (see Chang et al., *Nature* 275:615 (1978)), the trp promoter (Nichols and Yanofsky, *Meth in Enzymology* 101:155, (1983) and the tac promoter (Russell et al., *Gene* 20: 231, (1982)). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors

10 include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (Bolivar et al., *Gene* 2:9S, (1977)), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, *Meth in Enzymology* 101:20-77, 1983 and Vieira and Messing, *Gene* 19:259-268 (1982)), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La

15 Jolla, Calif.). Typical fusion expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ). Examples of inducible non-fusion expression vectors include pTrc (Amann et al., *Gene* 69:301-315 (1988)) and pET 11d (Studier et al., *Gene*

20 *Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California, 60-89 (1990)).

Yeast and fungi host cells suitable for carrying out the present invention include, but are not limited to *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*.

25 Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari. et al., *Embo J.* 6:229-234 (1987)), pMFa (Kurjan and Herskowitz, *Cell* 30:933-943 (1982)), pJRY88 (Schultz et al., *Gene* 54:113-123 (1987)), and pYES2 (Invitrogen Corporation, San Diego, CA). Protocols for the transformation of yeast and fungi are well known to those of ordinary skill

30 in the art.(see Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978); Itoh et al., *J. Bacteriology* 153:163 (1983), and Cullen et al. (*Bio/Technology* 5:369 (1987)).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651),

BHK (e.g. ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40), as well as other transcriptional and translational control sequences. Examples of mammalian expression vectors include pCDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman et al., *EMBO J.* 6:187-195 (1987)).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, the proteins of the invention may be expressed from plant cells (see Sinkar et al., *J. Biosci* (Bangalore) 11:47-58 (1987), which reviews the use of *Agrobacterium* rhizogenes vectors; see also Zambryski et al., *Genetic Engineering, Principles and Methods*, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York (1984), which describes the use of expression vectors for plant cells, including, among others, pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx*, *Trichoplusia* or *Spodoptera* species. Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., *Mol. Cell Biol.* 3:2156-2165 (1983)) and the pVL series (Lucklow, V.A., and Summers, M.D., *Virology* 170:31-39 (1989)). Some baculovirus-insect cell expression systems suitable for expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be expressed in non-human transgenic animals such as, rats, rabbits, sheep and pigs (Hammer et al. *Nature* 315:680-683 (1985); Palmiter et al. *Science* 222:809-814 (1983); Brinster et al. *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985); Palmiter and Brinster *Cell* 41:343-345 (1985) and U.S. Patent No. 4,736,866).

The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, *J. Am. Chem. Assoc.* 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, 5 *Methods of Organic Chemistry*, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart (1987)).

The present invention also provides proteins comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, 10 wherein the linker sequence contains a cleavage recognition site for a disease-specific protease. Such a protein could be prepared other than by recombinant means, for example by chemical synthesis or by conjugation of A and B chains and a linker sequence isolated and purified from their natural plant, fungal or bacterial source. Such A and B chains could be 15 prepared having the glycosylation pattern of the native ricin-like toxin.

N-terminal or C-terminal fusion proteins comprising the protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques. The resultant fusion proteins contain a protein of the invention fused to the 20 selected protein or marker protein as described herein. The recombinant protein of the invention may also be conjugated to other proteins by known techniques. For example, the proteins may be coupled using heterobifunctional thiol-containing linkers as described in WO 90/10457, N-succinimidyl-3-(2-pyridyldithio-propionate) or N-succinimidyl-5 25 thioacetate. Examples of proteins which may be used to prepare fusion proteins or conjugates include cell binding proteins such as immunoglobulins, hormones, growth factors, lectins, insulin, low density lipoprotein, glucagon, endorphins, transferrin, bombesin, asialoglycoprotein glutathione-S-transferase (GST), hemagglutinin (HA), 30 and truncated myc.

Utility of the Nucleic Acid Molecules and Proteins of the Invention

The proteins of the invention may be used to specifically inhibit or destroy mammalian cells affected by a disease or infection which have associated with such cells a specific protease, i.e., disease-specific, for

example cancer cells or cells infected with a virus, fungus or parasite, all of which are encompassed within the term "disease-specific." It is an advantage of the recombinant proteins of the invention that they have specificity for said cells without the need for a cell binding component. The ricin-like B chain of the recombinant proteins recognize galactose moieties on the cell surface and ensure that the protein is taken up by the diseased cell and released into the cytoplasm. When the protein is internalized into a non-infected cell, cleavage of the heterologous linker would not occur in the absence of the disease-specific protease and the A chain will remain inactive bound to the B chain. Conversely, when the protein is internalized into a diseased cell, the disease-specific protease will cleave the cleavage recognition site in the linker thereby releasing the toxic A chain.

The specificity of a recombinant protein of the invention may be tested by treating the protein with the disease-specific protease which is thought to be specific for the cleavage recognition site of the linker and assaying for cleavage products. Disease-specific proteases may be isolated from cancer cells or infected cells, or they may be prepared recombinantly, for example following the procedures in Darket et al. (*J. Biol. Chem.* 254:2307-2312 (1988)). The cleavage products may be identified for example based on size, antigenicity or activity. The toxicity of the recombinant protein may be investigated by subjecting the cleavage products to an *in vitro* translation assay in cell lysates, for example using Brome Mosaic Virus mRNA as a template. Toxicity of the cleavage products may be determined using a ribosomal inactivation assay (Westby et al., *Bioconjugate Chem.* 3:377-382 (1992)). The effect of the cleavage products on protein synthesis may be measured in standardized assays of *in vitro* translation utilizing partially defined cell free systems composed for example of a reticulocyte lysate preparation as a source of ribosomes and various essential cofactors, such as mRNA template and amino acids. Use of radiolabelled amino acids in the mixture allows quantitation of incorporation of free amino acid precursors into trichloroacetic acid precipitable proteins. Rabbit reticulocyte lysates may be conveniently used (O'Hare, *FEBS Lett.* 273:200-204 (1990)).

The ability of the recombinant proteins of the invention to selectively inhibit or destroy animal cancer cells or cells infected with a virus or parasite may be readily tested *in vitro* using animal cancer cell lines or cell cultures infected with the virus or parasite of interest. The selective inhibitory effect of the recombinant proteins of the invention may be determined, for example, by demonstrating the selective inhibition of viral antigen expression in infected mammalian cells, the selective inhibition of general mRNA translation and protein synthesis in diseased cells, or selective inhibition of cellular proliferation in cancer cells or infected cells.

Toxicity may also be measured based on cell viability, for example the viability of infected and non-infected cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

In another example, a number of models may be used to test the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a cancer-associated matrix metalloprotease. Thompson, E.W. et al. (*Breast Cancer Res. Treatment* 31:357-370 (1994)) has described a model for the determination of invasiveness of human breast cancer cells *in vitro* by measuring tumour cell-mediated proteolysis of extracellular matrix and tumour cell invasion of reconstituted basement membrane (collagen, laminin, fibronectin, Matrigel or gelatin). Other applicable cancer cell models include cultured ovarian adenocarcinoma cells (Young, T.N. et al. *Gynecol. Oncol.* 62:89-99 (1996); Moore, D.H. et al. *Gynecol. Oncol.* 65:78-82 (1997)), human follicular thyroid cancer cells (Demeure, M.J. et al., *World J. Surg.* 16:770-776 (1992)), human melanoma (A-2058) and fibrosarcoma (HT-1080) cell lines (Mackay, A.R. et al. *Lab. Invest.* 70:781-783 (1994)), and lung squamous (HS-24) and adenocarcinoma (SB-3) cell lines (Spiess, E. et al. *J. Histochem. Cytochem.* 42:917-929 (1994)). An *in vivo* test system involving the implantation of tumours and measurement of tumour growth and metastasis in athymic nude mice has also been described (Thompson, E.W. et al., *Breast Cancer Res. Treatment* 31:357-370 (1994); Shi, Y.E. et al., *Cancer Res.* 53:1409-1415 (1993)).

A further model may be used to test the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a cancer-associated Cathepsin B protease is provided in human glioma (Mikkelsen, T. et al. *J. Neurosurg*, 83:285-290 (1995)).

Similarly, the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a malarial protease may be tested by a Plasmodium invasion assay using human erythrocytes infected with mature-stage merozoite parasites as described by McPherson, R.A. et al. (*Mol. Biochem. Parasitol.* 62:233-242 (1993)). Alternatively, in vitro cultures of human hepatic parenchymal cells may be used to evaluate schizont infectivity and Plasmodium merozoite generation.

With respect to models of viral infection and replication, suitable animal cells which can be cultured *in vitro* and which are capable of maintaining viral replication can be used as hosts. The toxicity of the recombinant protein for infected and non-infected cultures may then be compared. The ability of the recombinant protein of the invention to inhibit the expression of these viral antigens may be an important indicator of the ability of the protein to inhibit viral replication. Levels of these antigens may be measured in assays using labelled antibodies having specificity for the antigens. Inhibition of viral antigen expression has been correlated with inhibition of viral replication (U.S. Patent No. 4,869,903). Toxicity may also be assessed based on a decrease in protein synthesis in target cells, which may be measured by known techniques, such as incorporation of labelled amino acids, such as [3H] leucine (O'Hare et al., *FEBS Lett.* 273:200-204 (1990)). Infected cells may also be pulsed with radiolabelled thymidine and incorporation of the radioactive label into cellular DNA may be taken as a measure of cellular proliferation. Toxicity may also be measured based on cell death or lysis, for example, the viability of infected and non-infected cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

Although the primary specificity of the proteins of the invention for diseased cells is mediated by the specific cleavage of the cleavage recognition site of the linker, it will be appreciated that specific cell binding components may optionally be conjugated to the proteins of the invention. Such cell binding components may be expressed as fusion proteins with the proteins of the invention or the cell binding component may be physically or chemically coupled to the protein component. Examples of suitable cell binding components include antibodies to cancer, viral or parasitic proteins.

Antibodies having specificity for a cell surface protein may be prepared by conventional methods. A mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g. the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., *Immunol. Today* 4:72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., *Monoclonal Antibodies in Cancer Therapy* Allen R., Bliss, Inc., pages 77-96 (1985)), and screening of combinatorial antibody libraries (Huse et al., *Science* 246:1275 (1989)). Hybridoma cells can be screened immunochemically for production of

antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a cell surface antigen (See, for example, Morrison et al., *Proc. Natl Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., E.P. Patent No. 171,496; European Patent No. 173,494, United Kingdom Patent No. GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g. Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today* 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982), and PCT Publication WO92/06193 or EP 239,400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with cell surface components. For example, complete Fab
5 fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., *Nature* 341:544-546 (1989); Huse et al., *Science* 246:1275-1281 (1989); and McCafferty et al., *Nature* 348:552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or
10 fragments thereof.

The proteins of the invention may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration in vivo" is meant a form of the
15 substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for
20 periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response.
25 For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The nucleic acid molecules of the invention may be formulated into pharmaceutical compositions for administration to subjects
30 in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration in vivo" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals.

Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary
5 according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated
10 by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, intramuscular, etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository applications. Depending
15 on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable
20 compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA
25 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions may be used in methods
30 for treating animals, including mammals, preferably humans, with cancer or infected with a virus or a parasite. It is anticipated that the compositions will be particularly useful for treating patients with B-cell lymphoproliferative disease, (melanoma), mononucleosis, cytomegalic inclusion disease, malaria, herpes, shingles, hepatitis, poliomyelitis, or

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infectious laryngotracheitis. The dosage and type of recombinant protein to be administered will depend on a variety of factors which may be readily monitored in human subjects. Such factors include the etiology and severity (grade and stage) of neoplasia, the stage of malarial infection (e.g. 5 exoerythrocytic *vs.* erythrocytic), or antigen levels associated with viral load in patient tissues or circulation.

As mentioned above, the novel recombinant toxic proteins and nucleic acid molecules of the present invention are useful in treating cancerous or infected cells wherein the cells contain a specific protease that 10 can cleave the linker region of the recombinant toxic protein. One skilled in the art can appreciate that many different recombinant toxic proteins can be prepared once a disease associated protease has been identified. For example, the novel recombinant toxic proteins and nucleic acid molecules of the invention may be used to treat CNS tumors. Muller et al. 15 (1993) describe increased activity of Insulin-type Growth Factor Binding Protein-3 (IGFBP-3) protease in the Cerebral Spinal Fluid of patients with CNS tumors. Cohen et al. (1992) claim that prostate-specific antigen (PSA) is an IGFBP-3 protease. The pAP290 construct described above is a substrate for PSA. Conover et al. (1994) claim that cathepsin D is IGFBP-3 20 protease. The pAP276 described herein is a substrate for cathepsin D. Another example of a specific use of the invention is treatment of human glioma which has been shown to produce cathepsin D (Mikkelsen, T. et al. *J. Neurosurg.*, 83:285-290 (1995)). The pAP 214 and 272 define herein are substrates for cathepsin B.

25 In addition, the novel proteins and nucleic acid molecules of the present invention may be used to treat cystic fibrosis. Hansen et al. (1995) describe how CF airway disease is characterized by neutrophil-dominated chronic inflammation with an excess of uninhibited neutrophil elastase (NE). NE levels in CF sputum are 350 times higher than that 30 found in normal sputum. The pAP294 described herein is a substrate for neutrophil elastase.

As well, the novel proteins and nucleic acid molecules of the present invention may also be used to treat multiple sclerosis. Bever Jr. et al. (1994) implicate cathepsin B (possibly from inflammatory cells of

hematogenous origin) in the demyelination found in multiple sclerosis. pAPs 214 and 272 defined herein present substrates for cathepsin B.

The term "animal" as used herein includes all members of the animal kingdom including mammals, preferably humans.

5 The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

Cloning and Expression of Proricin Variants Activated by Disease-Specific Proteases

10 Isolation of total RNA

Isolation of total RNA

 The preproricin gene was cloned from new foliage of the castor bean plant. Total messenger RNA was isolated according to established procedures (Sambrook et al., *Molecular Cloning: A Lab Manual* (Cold Spring Harbour Press, Cold Spring Harbour, (1989)) and cDNA generated using reverse transcriptase.

cDNA Synthesis:

 Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., Eur. J. Biochem., 145:266-270, 1985), several oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA synthesis was primed using the oligonucleotide Ricin1729C (Table 1). Three micrograms of total RNA was used as a template for oligo Ricin1729C primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

DNA Amplification and Cloning

30 The first strand cDNA synthesis reaction was used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA was amplified using the upstream primer Ricin-99 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular*

Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification was carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and
5 extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846bp amplified product was fractionated on an agarose gel (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the
10 manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA was then ligated (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RV-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene).
15 Positive clones were confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA was extracted using a Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

DNA Sequencing

The cloned PCR product containing the putative preproricin
20 gene was confirmed by DNA sequencing of the entire cDNA clone (pAP-144). Sequencing was performed using an Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB). The oligonucleotide primers used for sequencing were as follows: Ricin267,
25 Ricin486, Ricin725, Ricin937, Ricin1151, Ricini1399, Ricin1627, T3 primer (5'AATTAACCCTCACTAAAGGG-3') (SEQ ID NO. 128) and T7 primer (5'GTAATACGACTCACTATAGGGC-3) (SEQ ID NO. 129). Sequence data was compiled and analyzed using PC Gene software package (intelligenetics). The sequences and location of oligonucleotide primers is
30 shown in Table 1. The oligonucleotide primers shown in Table 1 have been assigned the following sequence ID numbers:

Ricin-109 is referred to herein as SEQ ID NO. 130;

Ricin-99Eco is referred to herein as SEQ ID NO. 131;

Ricin267 is referred to herein as SEQ ID NO. 132;

Ricin486 is referred to herein as SEQ ID NO. 133;

Ricin725 is referred to herein as SEQ ID NO. 134;

Ricin 937 is referred to herein as SEQ ID NO. 135;

Ricin 1151 is referred to herein as SEQ ID NO. 136;

5 Ricin 1399 is referred to herein as SEQ ID NO. 137;

Ricin 1627 is referred to herein as SEQ ID NO. 138;

Ricin 1729C is referred to herein as SEQ ID NO. 139; and

Ricin 1729C Xba is referred to herein as SEQ ID NO. 140.

Production and Cloning of Linker Variants

10 pAP144 cut with EcoRI was used as target for PCR pairs
employing the Ricin109-Eco oligonucleotide (Ricin-109Eco primer: 5-
GGAGGAATCCGGAGATGAAACCGGGAGGAAATACTATTGTAAT-3
(SEQ ID No. 141)) and a mutagenic primer for the 5' half of the linker as
well as the Ricin1729PstI primer (Ricin1729-PstI: 5-
15 GTAGGCGCTGCAGATAACTTGCTGTCCTTTCAG-3 (SEQ ID No. 142))
and a mutagenic primer for the 3' half of the linker. The cycling conditions
used for the PCRs were 98 degrees C for 2 min.; 98C 1 min., 52C 1 min.,
72C 1 min. 15 sec. (30 cycles); 72 degrees C 10min.; 4 degrees C soak. The
PCR products were then digested by EcoRI and PstI respectively,
20 electrophoresed on an agarose gel, and the bands purified by via glass
wool spin columns. Triple ligations comprising the PCR product pairs
(corresponding halves of the new linker) and pVL1393 vector digested
with EcoRI and PstI were carried out. Recombinant clones were identified
by restriction digests of plasmid miniprep DNA and the altered linkers
25 confirmed by DNA sequencing. See Figure 45 as an example of the cloning
strategy. Recombinant clones were identified by restriction digests of
plasmid miniprep DNA and the altered linkers confirmed by DNA
sequencing. Note that since all altered linker variants were cloned directly
into the pVL1393 vector odd-numbered pAPs were no longer required or
30 produced.

Isolation of Recombinant Baculoviruses

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and
BTI-TN-581-4 (High Five)) were maintained on EX-CELL 405 medium
(JRH Biosciences) supplemented with 10% total calf serum (Summers et al.,

09561451.041400
A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA was co-transfected with 0.5 microgram of BaculoGold AcNPV DNA (Pharmingen) into 2×10^6 Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants were then amplified by infecting Tn368 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 3 to 5 supernatants. A total of three rounds of amplification were performed for each recombinant following established procedures (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)).

20 Expression of Mutant Proricin

Recombinant baculoviruses were used to infect 1×10^7 Tn368 or sf9 cells at an moi of 9 in EX-CELL 405 media (JRH Biosciences) with 25mM α -lactose in spinner flasks. Media supernatants containing mutant proricens were collected 3 or 4 days post-infection.

25 EXAMPLE 2

Harvesting and affinity column purification of pro-ricin variants

Protein samples were harvested three days post transfection. The cells were removed by centrifuging the media at 8288 g for ten minutes using a GS3 (Sorvall) centrifuge rotor. The supernatant was further clarified by centrifuging at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes. Protease inhibitor phenylmethylsulfonyl fluoride (Sigma) was slowly added to a final concentration of 1mM. The samples were further prepared by adding lactose to a concentration of 20 mM (not including the previous lactose contained in the expression medium). The

samples were concentrated to 700 mL using a Prep/Scale-TFF Cartridge (2.5ft, 10K regenerated cellulose (Millipore)) and a Masterflex pump. The samples were then dialysed for 2 days in 1X Column Buffer (50 mM Tris, 100 mM NaCl, 0.02% NaN₃, pH 7.5) using dialysis tubing (10 K MWCO, 32 mm flat width(Spectra/Por)). Subsequently, the samples were clarified by centri fusing at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes.

Following centrifugation, the samples were degassed and applied at 4 degrees C to a XK26/20 (Pharmacia) column (attached to a Pharmacia peristaltic pump, Pharmacia Single-path Monitor UV-1 Control and Optical Units, and Bromma LKB 2210 2-Channel Recorder) containing 20 mL of a-Lactose Agarose Resin (Sigma). The column was washed for 3 hours with 1X Column buffer. Elution of pro-ricin variant was performed by eluting with buffer (1X Column buffer (0.1% NaN₃), 100 mM Lactose) until the baseline was again restored. The samples were concentrated using an Amicon 8050 concentrator (Amicon) with a YM10 76 mm membrane, utilizing argon gas to pressurize the chamber. The samples were further concentrated in Centricon 10 (Millipore) concentrators according to manufacturer's specifications.

Purification of Variant pAP-Protein by gel filtration chromatography

In order to purify the pro-ricin variant from processed material produced during fermentation, the protein was applied to a SUPERDEX 75 (16/60) column and SUPERDEX 200 (16/60) column (Pharmacia) connected in series equilibrated with 50 mM Tris, 100mM NaCl, pH 7.5 containing 100 mM Lactose and 0.1% β -mercaptoethanol (β ME). The flow rate of the column was 0.15 mL/min and fractions were collected every 25 minutes. The UV (280 nm) trace was used to determine the approximate location of the purified pAP-protein and thus determine the samples for Western analysis.

Western analysis of column fractions

Fractions eluted from the SUPERDEX columns (Pharmacia) were analyzed for purity using standard Western blotting techniques. An aliquot of 10 μ L from each fraction was boiled in 1X sample buffer (62.6 mM Tris-C1, pH 6.8, 4.4% β ME, 2% sodium dodecyl sulfate (SDS), 5% glycerol (all from Sigma) and 0.002% bromophenol blue (Biorad)) for five

minutes. Denatured samples were loaded on 12% Tris-Glycine Gels (Biorad) along with 50 ng of RCA₆₀ (Sigma) and 5 µL of kaleidoscope prestained standards (Biorad). Electrophoresis was carried out for ninety minutes at 100V in 25 mM Tris-Cl, pH 8.3, 0.1% SDS, and 192 mM glycine using the BioRad Mini Protean II cells (Biorad).

Following electrophoresis gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% Methanol) for a few minutes. PVDF Biorad membrane was presoaked for one minute in 100% methanol, rinsed in ddH₂O and two minutes in transfer buffer. Whatman paper was soaked briefly in transfer buffer. Five pieces of Whatman paper, membrane, gel, and another five pieces of Whatman paper were arranged on the bottom cathode (anode) of the Pharmacia Novablot transfer apparatus (Pharmacia). Transfer was for one hour at constant current (2 mA/cm²).

Transfer was confirmed by checking for the appearance of the prestained standards on the membrane. Non-specific sites on the membrane were blocked by incubating the blot for thirty minutes in 1X Phosphate Buffered Saline (1X PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) with 5% skim milk powder (Carnation). Primary antibody (Rabbit α-ricin, Sigma) was diluted 1:3000 in 1X PBS containing 0.1% Tween 20 (Sigma) and 2.5% skim milk and incubated with blot for forty five minutes on a orbital shaker (VWR). Non-specifically bound primary antibody was removed by washing the blot for ten minutes with 1X PBS containing 0.2% Tween 20. This was repeated four times. Secondary antibody donkey anti-rabbit (Amersham) was incubated with the blot under the same conditions as the primary antibody. Excess secondary antibody was washed as described above. Blots were developed with the ECL Western Blotting detection reagents according to the manufacturer's instructions. Blots were exposed to Medtec's Full Speed Blue Film (Medtee) or Amersham's ECL Hyperfilm (Amersham) for one second to five minutes. Film was developed in a KODAK Automatic Developer.

Determination of lectin binding ability of pro-ricin variant

An Immulon 2 plate (VDVR) was coated with 100 μ l per well of 10 μ g/ml of asialofetuin and left overnight at 4°C. The plate was washed with 3X 300 μ L per well with ddH₂O using an automated plate washer (BioRad). The plate was blocked for one hour at 37°C by adding 300 μ L per well of PBS containing 1% ovalbumin. The plate was washed again as above. Pro-ricin variant pAP-protein was added to the plate in various dilutions in 1X Baculo. A standard curve of RCA₆₀ (Sigma) from 1-10 ng was also included. The plate was incubated for 1 h at 37°C. The plate was washed as above. Anti-ricin monoclonal antibody (Sigma) was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin and 0.1% tween-20, added at 100 μ L per well and incubated for 1 h at 37°C. The plate was washed as above. Donkey-anti rabbit polyclonal antibody was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin, 0.1% Tween-20, and added at 100 μ L per well and incubated for 1 h at 37°C. The plate was given a final wash as described above. Substrate was added to plate at 100 μ L per well (1 mg/ml o-phenylenediamine (Sigma), 1 μ L/ml H₂O₂, 25 μ L of stop solution (20% H₂SO₄) was added and the absorbance read (A490nm-A630nm) using a SPECTRA MAX 340 plate reader (Molecular Devices).

Determination of pAP -Protein activity using the rabbit reticulocyte assay

- 20 Ricin samples were prepared for reduction.
- A) RCA₆₀ = 3,500 ng/ μ L of RCA₆₀ + 997 μ L 1xEndo buffer (25mM Tris, 25mM KCl, 5mM MgCl₂, pH 7.6)
Reduction = 95 μ L of 10ng/ μ L + 5 μ L β -mercaptoethanol
- B) Ricin variants
25 Reduction = 40 μ L variant + 2 μ L β -mercaptoethanol
The ricin standard and the variants were incubated for 30 minutes at room temperature.

Ricin - Rabbit Reticulocyte lysate reaction

The required number of 0.5 mL tubes were labelled. (2 tubes for each sample, + and - aniline). To each of the sample tubes 20 μ L of 1X endo buffer was added, and 30 μ L of buffer was added to the controls. To the sample tubes either 10 μ L of 10ng/ μ L Ricin or 10 μ L of

variant was added. Finally, 30µL of rabbit reticulocyte lysate was added to all the tubes. The samples were incubated for 30 minutes at 30°C using the thermal block. Samples were removed from the eppendorf tube and contents added into a 1.5 mL tube containing 1 mL of TRIZOL (Gibco).

5 Samples were incubated for 15 minutes at room temperature. After the incubation, 200 µL of chloroform was added, and the sample was vortexed and spun at 12,000 g for 15 minutes at 4°C. The top aqueous layer from the samples was removed and contents added to a 1 mL tube containing 500 µL of isopropanol. Samples were incubated for 15 minutes at room

10 temperature and then centrifuged at 12,000 for 15 minutes at 4°C. Supernatant was removed and the pellets were washed with 1 mL of 70% ethanol. Centrifugation at 12,000 g for 5 minutes at 4°C precipitated the RNA. All but approximately 20 µL of the supernatant was removed and air dried. Pellets from the other samples (+aniline samples) were dissolved

15 in 20 µL of DEPC treated ddH₂O. An 80 µL aliquot of 1 M aniline (distilled) with 2.8 M acetic acid was added to these RNA samples and transferred to a fresh 0.5 mL tube. The samples were incubated in the dark for 3 minutes at 60°C. RNA was precipitated by adding 100 µL of 95% ethanol and 5µL of 3M sodium acetate, pH 5.2 to each tube and centrifuging at 12,000 g for

20 30 minutes at 4°C. Pellets were washed with 1 mL 70% ethanol and centrifuged again at 12,000g for 5 minutes at 4°C to precipitate RNA. The supernatant was removed and air dried. These pellets were dissolved in 10µL of 0.1 X E buffer. To all samples, 10 µL of formamide loading dye was added. The RNA ladder (8 µL of ladder + 8 µL of loading dye) was

25 also included. Samples were incubated for 2 minutes at 70°C on the thermal block. Electrophoresis was carried out on the samples using 1.2% agarose, 50% formamide gels in 0.1X E buffer + 0.2% SDS. The gel was run for 90 minutes at 75 watts. RNA was visualized by staining the gel in 1 µg/µL ethidium bromide in running buffer for 45 minutes. The gel was

30 examined on a 302 nm UV box, photographed using the gel documentation system and saved to a computer disk.

Results:

Protein Expression Yields

Aliquots were taken at each stop of the harvesting/purification and tested. Yields of functional ricin variant were determined by ELISA. Typical results of an 2400 mL prep of infected *T. ni* cells are given below.

5	<u>Aliquot</u>	<u>µg pAP 220</u>
	Before concentration and dialysis	6000
	After concentration and dialysis	4931
	alpha- Lactose agarose column flow through	219
10	alpha- Lactose agarose column elution	1058

Yield: $1058/6000 = 17.6\%$

Purification of pAP -Protein and Western Analysis of column fractions

Partially purified pAP-protein was applied to Superdex 75 and 200 (16/60) columns connected in series in order to remove the contaminating non-specifically processed pAP-protein. Eluted fractions were tested via Western analysis as described above and the fractions containing the most pure protein were pooled, concentrated and re-applied to the column. The variant was applied a total of three times to the column. Final purified pAP-protein has less than 1% processed variant.

The purified pAP-protein was tested for susceptibility to cleavage by the particular protease and for activation of the A-chain of the pro-ricin variant, (inhibition of protein synthesis). Typically, pAP-protein was incubated with and without protease for a specified time period and then electrophoresed and blotted. Cleaved pAP will run as two 30 kDa proteins (B is slightly larger) under reducing (SDS-PAGE) conditions. Unprocessed pAP-protein, which contains the linker region, will run at 60 kDa.

Activation of pAP -Protein variant with Specific Protease

Activation of protease treated pAP-protein is based on the method of May *et al.* (EMBO Journal. 8 301-8, 1989). Activation of ricin A chain upon cleavage of the intermediary linker results in catalytic depurination of the adenosine 4325 residue of 28S or 26S rRNA. This depurination renders the molecule susceptible to amine-catalyzed hydrolysis by aniline of the

- phosphodiester bond on either side of the modification site. The result is a diagnostic 390 base band. As such, reticulocyte ribosomes incubated with biochemically purified ricin A chain, released the characteristic RNA fragment upon aniline treatment of isolated rRNA (May, M.J. et al. *Embo. Journal*, 8:301-308 at 302-303 (1989)). It is on this basis that the assay allows for the determination of activity of a ricin A chain which has been cleaved from the intact unit containing a particular variant linker sequence.

EXAMPLE 3

In Vitro Protease Digestion of Proricin Variants:

- 10 Affinity-purified proricin variant is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. Ricin-like toxin variants are eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Proricin substrate is then
- 15 incubated at 37°C for 60 minutes with a disease-specific protease. The cleavage products consisting ricin A and B chains are identified using SDS/PAGE (Sambrook et al., *Molecular Cloning: a Laboratory Manual*, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).
- 20 Cathepsin B may be obtained from Medcor or Calbiochem. Matrix metalloproteinases may be prepared substantially as described by Lark, M.W. et al. (*Proceedings of the 4th International Conference of the Inflammation Research Association* Abstract 145 (1988)) and Welch, A.R. et al. (*Arch. Biochem. Biophys.* 324:59-64 (1995)). Candida acid protease may be
- 25 prepared substantially as described in Remold, H.H. et al. (*Biochim. Biophys. Acta* 167:399-406 (1968)), Ray, T.L. and Payne, C.D. (*Infect. Immunol.* 58:508-514 (1990)) and Fusek, M. et al. (*FEBS Lett.* 327:108-112 (1993)). Hepatitis A protease may be prepared as described in Jewell, D.A. et al. (*Biochemistry* 31:7862-7869 (1992)). Plasmodium proteases may be prepared as described
- 30 in Goldberg, D.E. et al. (*J. Exp. Med.* 173:961-969 (1991)) and Cooper, J.A. and Bujard, H. (*Mol. Biochem. Parasitol.* 56:151-160 (1992)).

In Vitro Cytotoxicity Assay:

Human ovarian cancer cells (e.g. MA148) are seeded in 96-well flat-bottom plates and are exposed to ricin-like toxin variants or control

medium at 37°C for 16 h. The viability of the cancer cells is determined by measuring [³⁵S]methionine incorporation and is significantly lower in wells treated with the toxin variants than those with control medium.

In Vivo Tumour Growth Inhibition Assay:

- 5 Human breast cancer (e.g. MCF-7) cells are maintained in suitable medium containing 10% fetal calf serum. The cells are grown, harvested and subsequently injected subcutaneously into ovariectomized athymic nude mice. Tumour size is determined at intervals by measuring two right-angle measurements using calipers. In animals that received ricin-
10 like toxin variants containing the matrix metalloproteinase-sensitive linkers, tumour size and the rate of tumour growth are lower than animals in the control group.

In Vivo Tumour Metastasis Assay:

- The metastasis study is performed substantially as described in
15 Honn, K.V. et al. (*Biochem. Pharmacol.* 34:235-241 (1985)). Viable B16a melanoma tumour cells are prepared and injected subcutaneously into the left axillary region of syngeneic mice. The extent of tumour metastasis is measured after 4 weeks. The lungs are removed from the animals and are fixed in Bouin's solution and macroscopic pulmonary metastases are
20 counted using a dissecting microscope. In general without therapeutic intervention, injection of 10⁵ viable tumour cells forms approximately 40-50 pulmonary metastases. The number of metastases in animal treated with proricin variants containing cathepsin B-sensitive linkers is substantially lower.

25 EXAMPLE 4

In Vitro Protease Digestion of Proricin Variants by Cancer Proteases Cathepsin B or MMP-9

The general protocol for proricin digestion by cancer proteases is described in Examples 2 and 3.

30 In Vitro Protease Digestion of Cathepsin B Proricin Variant

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The proricin substrate is digested in a Cathepsin B protease buffer (50 mM Sodium acetate, 2 mM EDTA, 0.05% Triton) at 40°C. Two hours and

overnight (16 hr) digestion reactions are carried out using 100ng of proricin substrate and 100 and 618 ng of Cathepsin B protease per reaction (CALBIOCHEM, USA). The cleavage products of proricin (ricin A and B chains) are identified using SDS/PAGE (Sambrook et al., Molecular cloning: a laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

In Vitro Protease Digestion of MMP-9 Proricin Variant

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The proricin substrate is digested in 1X column buffer (100 mM NaCl, 50 mM Tris, PH 7.5) at 37°C. Two hours and overnight (16 hr) digestion reactions are set up using 50 ng of MMP-9 proricin substrate and 20 and 200 ng of MMP-9 protease per reaction (CALBIOCHEM, USA). The cleavage products of proricin (ricin A and B chains) are identified using SDS/PAGE (Sambrook et al., Molecular cloning: a laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

The protocol for Western analysis of ricin chains is described in Example 2.

Results

Figures 48 and 49 illustrate Western blots showing the cleavage of the protease-sensitive linkers by cathepsin B (pAP 214) and MMP-9 (pAP 220) respectively. Without protease digestion, the proricin variant appears as a single band at approximately 60 kDa (Lane B of Figure 48 and Lane A of Figure 49). Wild type ricin A chain and B chain appear as two disparate bands at approximately 30 kDa (Lane A of Figure 48 and Lane E of Figure 49). Increasing extent of proricin cleavage can clearly be observed with increasing protease concentration (Lanes C and D of Figure 48 and Lanes B-C of Figure 49).

EXAMPLE 5

In vitro protease digestion of various proricin variants by their corresponding proteases.

The general protocol for proricin digestion by corresponding proteases was as described in Examples 2 and 3 and should be considered in connection with the digestions described below.

Cleavage of pAP-222 protein with the Matrix Metalloproteinase 2

5 **(MMP-2)**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-222 protein sample (1.0 ug) was digested with the MMP-2 protease (1.0 ug) overnight at 37° C. The total volume of the digestion
10 reaction was 21.5 ul, and 0.250 ug of the reaction sample was loaded on a protein gel. The MMP-2 protease was purchased from Calbiochem-Novabiochem Corporation, USA.

Cleavage of pAP-248 protein with the Human Cytomegalovirus (HCMV) protease

15 Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-248 protein sample (1.19 ug) was digested with the HCMV protease (1.13 ug) overnight at 37°C. The total volume of the digestion was 10.5 ul, and 0.279 ug of the reaction sample was loaded on a protein gel.
20 The HCMV was purchased from BACHEM Bioscience Inc., USA.

Cleavage of pAP-256 protein with the Hepatitis A virus 3C (HAV 3C) protease

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

25 The pAP-256 protein sample (1.26 ug) was digested with the HAV 3C protease (5 ug) overnight at 37°C. The total volume of the digestion was 12.5 ul, and 0.302 ug of the digestion sample was loaded on a protein gel. The HAV 3C protease was a gift from Dr. G. Lawson from Bates Collage, Main, USA.

30 **Cleavage of pAP-270 protein with the Matrix Metalloproteinase 2 (MMP-2)**

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.

The pAP-270 protein sample (0.120 ug) was digested with the MMP-2 protease (0.25 ug) overnight at 37° C. The total volume of the digestion reaction was 22.5 ul, and 0.106 ug of the reaction sample was loaded on a protein gel. The MMP-2 protease was purchased from
5 Calbiochem-Novabiochem Corporation, USA.

Cleavage of pAP-288 protein with tPA plasminogen tissue activator

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The pAP-288 protein sample (1.65 ug) was digested with the t-PA protease (0.5
10 ug) overnight at 37° C. The total volume of the digestion reaction was 55 ul, and 0.6 ug of the reaction sample was loaded on a protein gel. The t-PA was purchased from Sigma Chemical Co., USA.

Cleavage of pAP-294 protein with human neutrophil elastase

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region.
15 The pAP-256 protein sample (0.6 ug) was digested with the Elastase protease (5 ug) at 25° C for one hour. The total volume of the digestion reaction was 52.5 ul, and 0.171 ug of the digestion sample was loaded on a protein gel. The Human Neutrophil Elastase protease was purchased from
20 Cedarlane Laboratories Limited, Canada.

Cleavage of pAP-296 protein with calpain

Affinity-purified mutant proricin is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. The pAP-296 protein sample (2.05 ug) was digested with the Calpain protease
25 (10 ug) overnight at 37° C. The total volume of the digestion reaction was 35 ul and 0.761 ug of the reaction sample was loaded on a protein gel. The Calpain protease was purchased from Sigma Chemical Co., USA

Results

Figures 52, 54, 58 & 66(MMP-2), 60, 64 and 62 show the cleavage of
30 proteases of linkers by HCMV, HAV 3C, MMP-2, t-PA, calpain, and human neutrophil elastase respectively. Without protease digestion, the proricin variants appear as a single band at approximately 60kDA (Lane A in connection with Figure 52; Lane B of Figure 54; Lane A of Figure 58; Lane B of Figure 60; and Lane C of Figure 62; lane B of Figure 64 and lane B of

Figure 66). Wild type ricin chain A and B appear as two bands at approximately 30kDA (see for example Lanes C and D of Figure 52) proricin cleavage can clearly be observed with the appearance of 30kDA bands in connection with the protein which has been digested by the
5 respective protease (see Lane B of Figure 52; Lane C of Figure 54; or Lane B of Figure 58 for examples).

EXAMPLE 6

In Vitro Translation Assay (Activation by Cancer Proteases Cathepsin B or MMP-9)

10 The general protocol for the rabbit reticulocyte lysate reaction to test the cytotoxicity of cancer protease-activated proricin is described briefly in Example 3 and is described in more detail in Example 2.

Results

Activation of pAP 214 and pAP 220 proricin variants by cathepsin B
15 and MMP-9, based on the method of May et al. (EMBO J. 8:301-308, 1989), is illustrated in Figures 50 and 51 respectively. The appearance of the 390 base pair product (positive control) is observed in Lane F of Figure 50 and Lane G of Figure 51. This 390 base pair product is absent in the negative control lanes. Without cathepsin or MMP-9 activation, no or minimal N-glycosidase activity in the pAP 214 variant (Lanes H to L, Figure 50) or the
20 pAP 220 variant (Lanes A to E, Figure 51) was observed. When the pAP 214 variant and the pAP 220 variant were activated by cathepsin or MMP-9 respectively, appearance of the 390 base pair product was observed in a proricin concentration-dependent manner (Lanes A to E of Figure 50 and
25 Lanes H to L of Figure 51). The present experimental series demonstrated the successful and selective activation of proricin variants by cancer-associated proteases.

EXAMPLE 7

The general protocol for the rabbit reticulocyte lysate reaction is
30 described briefly in Example 3 and is described in more detail in Example 2, all of which compliments the description below.

Depurination of Rabbit Reticulocyte 28S Ribosomal RNA by Digested and Undigested Ricin Variants

Affinity-purified mutant proricin mutants which were previously digested with the disease-specific protease, were reduced with 5% 2-mercaptoethanol then diluted to 100ng, 14.2ng, 2.0ng, 291pg, and 41.7pg with 1 X ENDO buffer (25mM Tris pH 7.6, 25mM KCl, 5mM MgCl₂) and
5 incubated with rabbit reticulocyte lysate, untreated (Promega) for 30 minutes at 30°C. To compare the digested with the undigested proricin variant, the proricin in digestion buffer (according to the specific digestion protocol) was treated in the same manner as the digested sample. As a positive and negative control, 10ng of ricin A chain and 1 X ENDO buffer
10 consecutively, was incubated with rabbit reticulocyte lysate, untreated, for 30 min at 30°C.

Aniline Cleavage of rRNA and Gel Fractionation

Total RNA was then extracted from reticulocyte lysate translation mixtures with Trizol reagent (Gibco-BRL) as per manufacturer's
15 instructions. The RNA was incubated with 80ul of 1M aniline (distilled) with 2.8M acetic acid for 3 min at 60°C in the dark. Ethanol-precipitated RNA samples were dissolved in 20ul of 50% formamide, 0.1X E buffer (3.6mM Tris, 3mM NaH₂PO₄, 0.2mM EDTA), and 0.05% xylene cyanol. 10ul of this was heated to 70°C for 2 minutes, loaded and electrophoresed
20 in 1.2% agarose, 0.1X E buffer, and 50% formamide gel with RNA running buffer (0.1 X E buffer, 0.2% SDS).

Results

Activation of pAP-248 proricin variant by HCMV; pAP-256 by HAV3C protease; pAP-270 by MMP-2 protease; pAP-288 by t-PA protease;
25 pAP-294 by human neutrophil elastase; pAP-296 by calpain; and pAP-222 by MMP-2 is illustrated in Figures 52, 55, 59, 61, 63, 65, and 67 respectively. The appearance of the 390 base pair product (deposit of control) is observed in lane L of Figures 53, 55, 61, 63, 65 and 67. The 390 base pair product is observed in lane A of Figures 59 (activation of pAP-270 by
30 MMP-2). This 390 base pair product is absent in the negative control lanes. Without the specific protease activation, no or minimal activity is seen in the lanes which contained only the proricin variant without digestion (see lane A, B, C, D, and E of Figures 53, 55, 61, 63, 65, and 67). The same observation is made in connection with pAP-270 in Figure 59, however, the

undigested lanes appear as H, I, J, K and L. When the variant was activated by its respective protease, there is an appearance of the 390 base pair product in a proricin concentration-dependent manner (see Lanes H, I, J, K and L of Figure 53, 55, 61, 63, 65, and 67 and Lanes A, B, C, D, and E of Figure 59). The present experimental series demonstrate the successful and selective activation of the identified proricin variants by selective corresponding proteases.

EXAMPLE 8

Procedure for Examining the Cytotoxicity of Ricin and Ricin Variants on the COS-1 Cell Line

Cell Preparation

After washing with 1XPBS (0.137 M NaCl, 2.68 mM KCl, 8.10 mM Na₂HPO₄, 1.47 mM KH₂PO₄), cells in log phase growth were removed from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were centrifuged at 1100 rpm for 3 min, resuspended in Dulbecco's Modified Eagle Medium containing 10%FBS and 1X pen/strep, and then counted using a haemocytometer. They were adjusted to a concentration of 5 X 10⁴ cells•ml⁻¹. One hundred microliters per well of cells was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well tissue culture plate. A separate 96 well tissue culture plate was used for each sample of Ricin or Ricin variant. The plates were incubated at 37°C with 5% CO₂ for 24 hours.

Toxin Preparation

The Ricin and Ricin variants were sterile filtered using a 0.22µm filter (Millipore). The concentration of the sterile samples were then quantified by A₂₈₀ and confirmed by BCA measurements (Pierce). For the variants digested with the protease in vitro, the digests were carried out as described in the digestion procedure for each protease. The digests were then diluted in the 1000 ng•ml⁻¹ dilution and sterile filtered. The Ricin and the undigested pAP214 in the pAP 214 cytotoxicity data were treated in the same manner but without the Cathepsin B treatment. Ricin and Ricin variants were serially diluted to the following concentrations: 1000 ng•ml⁻¹, 100 ng•ml⁻¹, 10 ng•ml⁻¹, 1 ng•ml⁻¹, 0.1 ng•ml⁻¹, 0.01 ng•ml⁻¹, 0.001 ng•ml⁻¹ with media containing 10%FBS and 1X pen/strep.

Application of Toxin or Variants to Plates

5
10

Sample Application

15
20

Results

25
30

EXAMPLE 9

Procedure for Examining the Cytotoxicity of Ricin and Ricin Variants on Various Tissue Culture Cell Lines

Cell Preparation

After washing with 1XPBS (1.37M NaCl, 26.8mM KCl, 81mM Na₂HPO₄, 14.7mM KH₂PO₄), cells in log phase growth were removed from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were
5 centrifuged at 1100 rpm for 3 min, resuspended in media containing 10%FBS and 1X pen/strep (media used depended on the cell line being tested), and then counted using a haemocytometer. They were adjusted to a concentration of 5×10^4 cells•ml⁻¹ (faster growing cell lines were adjusted to 2×10^4 cells•ml⁻¹). One hundred microliters per well of cells
10 was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well tissue culture plate. A separate 96 well tissue culture plate was used for each sample of Ricin or Ricin variant. The plates were incubated at 37(C with 5% CO₂ for 24 hours.

Toxin Preparation

15 The Ricin and Ricin variants were sterile filtered using a 0.22µm filter (Millipore). The concentration of the sterile samples were then quantified by A₂₈₀ and confirmed by a BCA measurement (Pierce). Ricin and Ricin variants were serially diluted to the following concentrations: 3000 ng•ml⁻¹, 300 ng•ml⁻¹, 30 ng•ml⁻¹, 3 ng•ml⁻¹, 0.3 ng•ml⁻¹,
20 0.03ng•ml⁻¹, 0.003 ng•ml⁻¹ with media containing 10%FBS and 1X pen/strep.

Application of Toxin or Variants to Plates

Columns 2 to 9 were labeled: control, 0.001 ng•ml⁻¹, 0.01 ng•ml⁻¹, 0.1 ng•ml⁻¹, 1ng•ml⁻¹, 10 ng•ml⁻¹, 100 ng•ml⁻¹, 1000 ng•ml⁻¹
25 consecutively. For each plate of variant and toxin, 50µl of media was added to wells 2B to 2G as the control, and 50µl of each sample dilution was added to the corresponding columns containing 100µl per well of cells (i.e. 50 µl of the 3000 ng•ml⁻¹ dilution added to the wells B-G in column 9, labeled 1000 ng•ml⁻¹). The plates were incubated for 48 hours at 37(C
30 with 5% CO₂.

Sample Application

An amount of 140µl was removed from each well with a multichannel pipettor, and replaced with 100 µl of the substrate mixture

(Promega Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit). The plates were incubated at 37°C with 5% CO₂ for 2 to 4 hours, and subsequently read with a Spectramax 340 96 well plate reader at 490nm. The IC₅₀ values were calculated using the GRAFIT software program.

5 **Results**

Referring to Table 2, it may be seen that the survival of cells is correlated with the proricin variant and the cell specific protease produced by the cell type. For example, in the HT1080 cell line, both pAP-214 and pAP-220 required only 2-1/2 times the amount of ricin to achieve the same level of cytotoxicity. On the other hand, pAP-224 required 193 times the amount of ricin to achieve the same level of cell death. As well, it may be seen that in the cells where expression of Cathepsin D is found, pAP-214 and 220 were more effective at causing cell death than ricin and more effective than pAP-224. Details concerning the various cells types used in these experiments are outlined below.

COS-1 (African Green Monkey Kidney Cells)

This is an SV40 transformed cell line which was prepared from established simian cells CV-1. (Reference: Gluzman, Y. (1975) Cell, 23, 175 - 182)(ATCC CRL 1650)

20 **HT-1080 Human Fibrosarcoma**

(ATCC CCL 121) This cell line was shown to produce active MMP-9 in tissue culture. References: Moore et al. (1997) Gynecologic Oncology 65, 83-88.

9L Rat Glioblastoma

25 Glioblastomas are generally associated with cathepsin B expression. Levels of cathepsin B expression correspond to the extent of progression of malignancy i.e. highest levels for glioblastomas over anaplastic astrocytomas over low-grade gliomas and normal brain tissue. The 9L cell line was provided by Dr. William Jia of the B.C. Cancer Agency.

30 References: Mikkelsen et al. (Aug. 1995) Journal of Neurosurgery 83(2), 285-290. Nakano et al. (1995) J. of Neurosurgery 83(2), 298-307.

MCF-7 Human Breast Cancer Cell Line (Epithelial)

(ATCC CRL 1555) In the absence of estrogen cathepsin B has not been shown to be elevated relative to normal cells. It can be induced with estrogen to produce Cathepsin D. Production of MMP-9 is unknown.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Bever Jr., C.T., Panitch, H.S., and Johnson, K.P. (1994) *Neurology* 44(4), 745-8. Increased cathepsin B activity in peripheral blood mononuclear cells of multiple sclerosis patients.

Cohen, P., Graves, H.C., Peehl, D.M., Kamarei, M., Giudice, L.C., and Rosenfeld, R.G. (1992) Journal of Clinical Endocrinology and Metabolism 75(4), 1046-53. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma.

- 10 Conover, C.A. and De Leon, D.D. (1994) J. Biol. Chem. 269(10), 7076-80.
Acid activated insulin-like growth factor-binding protein-3 proteolysis in
normal and transformed cells. Role of cathepsin D.

Hansen, G., Schuster, A., Zubrod, C., and Wahn, V. (1995) Respiration
62(3), 117-24. Alpha 1-proteinase inhibitor abrogates proteolytic and
15 secretagogue activity of cystic fibrosis sputum.

Muller, H.L., Oh, Y., Gargosky, S.E., Lehrnbecher, T., Hintz, R.L., and Rosenfeld, R.G. (1993) Journal of Clinical Endocrinology and Metabolism 77(5), 1113-9. Concentrations of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), IGF, and IGFBP-3 protease activity in cerebrospinal fluid of children with leukemia, central nervous system tumor, or meningitis.

TABLE 1

Table 1 - Sequence and Location of Oligonucleotide Primers

Name of Primer	Primer Sequence †	Corresponds to preproricin nucleotide numbers: (see Figures 8-10)
Ricin-109	5' - GGAGATGAAACCGGGAGGAAATACTATTGTAAT-3'	27 to 59
Ricin-99Eco	5' - <u>GCGGAATT</u> CCGGGAGGAAATACTATTGTAAT -3'	37 to 59
Ricin267	5' - ACGGTTTATTTTAGTTGA-3'	300 to 317
Ricin486	5' - ACTTGCTGGTAATCTGAG -3'	519 to 536
Ricin725	5' - AGAATAGTTGGGGGAGAC -3'	758 to 775
Ricin937	5' - AATGCTGATGTTTGTATG -3'	970 to 987
Ricin1151	5' - CGGGAGTCTATGTGATGA -3'	1184 to 1201
Ricin1399	5' -GCAAATAGTGGACAAGTA -3'	1432 to 1449
Ricin 1627	5' - GGATTGGTGTTAGATGTG -3'	1660 to 1677
Ricin1729C	5' - ATAACCTGCTGTCCTTTCA -3'	1864 to 1846
Ricin1729C Xba	5' - <u>CGCTCTAG</u> AATACTTGCTGTCCTTTCA	1864 to 1846

† Underlined sequences inserted for subcloning purposes and not included in final preproricin sequences

00440" 634360

<u>Cell Line</u>	<u>IC50_{Ricin}</u> <u>(ng/ml)</u>	<u>IC50_{pAP214}</u> <u>IC50_{Ricin}</u>	<u>IC50_{pAP220}</u> <u>IC50_{Ricin}</u>	<u>IC50_{pAP224}</u> <u>IC50_{Ricin}</u>
COS-1	0.1	17	22	150
HT1080	0.5	2.46	2.14	193
9L	10.8	1.3	1.7	32.3
MCF-7 (without estrogen)	0.09	27.8	40	742

<u>Cell Line</u>	<u>IC50_{Ricin}</u> <u>(ng/ml)</u>	<u>IC50_{pAP214}</u> <u>IC50_{Ricin}</u>	<u>IC50_{pAP220}</u> <u>IC50_{Ricin}</u>	<u>IC50_{pAP224}</u> <u>IC50_{Ricin}</u>
COS-1	0.1	17	22	150
HT1080	0.5	2.46	2.14	193
9L	10.8	1.3	1.7	32.3
MCF-7 (without estrogen)	0.09	27.8	40	742

I CLAIM:

1. A purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, the
5 heterologous linker sequence containing a cleavage recognition site for a protease localized in cells or tissues affected by a specific disease.
2. The nucleic acid sequence of claim 1 wherein the linker sequence encodes a peptide wherein at least 5 amino acids differ from the wild-type.
3. The nucleic acid sequence of claim 2 wherein the linker sequence
10 contains a cleavage recognition site recognized by a protease selected from the group consisting of: a cancer associated protease, a viral protease, a fungal protease, and a parasite protease.
4. A nucleic acid sequence of claim 3 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, or Domain I of
15 Pseudomonas exotoxin.
5. A nucleic acid molecule of claim 3 wherein the A chain is volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain or shiga toxin A chain.
6. A nucleic acid sequence of claim 3 wherein the B chain is ricin B
20 chain, abrin toxin A chain, diphtheria toxin B chain, or Domain II/III of Pseudomonas exotoxin.
7. A nucleic acid molecule of claim 3 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain or shiga toxin B chain.
- 25 8. A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by a cancer-associated protease which is selected from

the group consisting of: cathepsin B, a matrix metalloproteinase, cathepsin L, cathepsin D, urokinase-type plasminogen activator, tissue-type plasminogen activator, human prostate-specific antigen, kallikrein, neutrophil elastase, and calpain.

- 5 9. A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by a parasitic protease which is a *Plasmodium falciparum* protease.

- 10 10. A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by viral protease which is selected from the group consisting of: human cytomegalovirus, human herpes virus, varicella zoster virus, hepatitis A virus, hepatitis C virus, Epstein-Barr virus-specific protease, and infectious laryngotracheitis virus.

11. A nucleic acid sequence of claim 3 wherein the cleavage recognition site is recognized by fungal protease which is a *Candida* acid protease.

- 15 12. A nucleic acid sequence of claim 3 having the nucleotide sequence according to SEQ ID No. 3; SEQ ID No 5; SEQ ID No 7; SEQ ID No 9; SEQ ID No 11; SEQ ID No 13; SEQ ID No 15; SEQ ID No 17; SEQ ID No 19; SEQ ID No 21; SEQ ID No 23; SEQ ID No 25; SEQ ID No 27; SEQ ID No 29; SEQ ID No 31; SEQ ID No 33; SEQ ID No 35; SEQ ID No 37; SEQ ID No 39; 20 SEQ ID No 48; SEQ ID No 50; SEQ ID No 52; SEQ ID No 54; SEQ ID No 74; SEQ ID No 77; SEQ ID No 80; SEQ ID No 83; SEQ ID No 86; SEQ ID No 89; SEQ ID No 92; SEQ ID No 95; SEQ ID No 98; SEQ ID No 101; SEQ ID No 104; SEQ ID No 107; SEQ ID No 110; SEQ ID No 113; SEQ ID No 116; SEQ ID No 119; SEQ ID No 122; or SEQ ID No 125.

- 25 13. A plasmid incorporating the nucleic acid of claim 12.

14. A baculovirus transfer vector incorporating the nucleic acid of claim 12.

15. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a protease localized in cells or tissues affected by a specific disease.
16. The recombinant protein of claim 15 wherein at least 5 amino acids of the linker amino acid sequence differ from the wild-type.
17. The recombinant protein of claim 16 wherein the linker sequence contains a cleavage recognition site which is recognized by a protease selected from the group consisting of: a cancer, viral, fungal, and a parasitic protease.
18. A recombinant protein of claim 17 wherein the A chain is ricin A chain, abrin toxin B chain, diphtheria toxin A chain, or Domain I of Pseudomonas exotoxin.
19. A recombinant protein of claim 17 wherein the A chain is volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain, viscumin toxin A chain, or shiga toxin A chain.
20. A recombinant protein of claim 17 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, or Domain II/III of Pseudomonas exotoxin.
21. A recombinant protein of claim 17 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain, viscumin toxin B chain, or shiga toxin B chain.
22. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a cancer-associated protease selected from the group consisting of: cathepsin B, a matrix metalloproteinase, cathepsin L, cathepsin D, urokinase-type plasminogen activator, tissue-type

plasminogen activator, human prostate-specific antigen, kallikrein, neutrophil elastase, and calpain.

23. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a parasitic protease which is a
5 Plasmodium falciparum protease.

24. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a viral protease which is selected from the group consisting of: human cytomegalovirus, human herpes virus, varicella zoster virus, hepatitis A virus, hepatitis C virus, Epstein-Barr
10 virus-specific protease, and infectious laryngotracheitis virus.

25. A recombinant protein of claim 17 wherein the cleavage recognition site is recognized by a fungal protease which is a Candida acid protease.

26. A recombinant protein of claim 17 having the linker amino acid
15 sequence according to SEQ ID No. 40; SEQ ID No. 41; SEQ ID No. 42; SEQ ID No. 43; SEQ ID No. 44; SEQ ID No. 45; SEQ ID No. 46; SEQ ID No. 55; SEQ ID No. 56; SEQ ID No. 57; SEQ ID No. 58; SEQ ID No. 59; SEQ ID No. 60; SEQ ID No. 61; SEQ ID No. 62; SEQ ID No. 63; SEQ ID No. 64; SEQ ID No. 65; SEQ ID No. 66; SEQ ID No. 67; SEQ ID No. 68; SEQ ID No. 69; SEQ
20 ID No. 70; SEQ ID No. 71; SEQ ID No. 72; SEQ ID No. 75; SEQ ID No. 78; SEQ ID No. 81; SEQ ID No. 84; SEQ ID No. 87; SEQ ID No. 90; SEQ ID No. 93; SEQ ID No. 96; SEQ ID No. 99; SEQ ID No. 102; SEQ ID No. 105; SEQ ID No. 108; SEQ ID No. 111; SEQ ID No. 114; SEQ ID No. 117; SEQ ID No. 120; SEQ ID No. 123; or SEQ ID No. 126.

25 27. A method of inhibiting or destroying cells of a tissue affected by a disease, wherein a protease specific to the disease is localized in the cells or tissue comprising the steps of:

(a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a

ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease;

(b) introducing the nucleic acid into a host cell and expressing the
5 nucleic acid in the host cell to obtain a recombinant protein comprising an
A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino
acid sequence;

(c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient, and

10 (d) contacting the cells with the recombinant protein.

28. A method according to claim 27 wherein the linker sequence encodes a peptide wherein at least 5 amino acids differ from the wild-type.

29. The method of claim 28 where the disease is one of cancer, a fungal infection, or cells infected with a virus or parasite.

15 30. A method of inhibiting or destroying cells of a tissue affected by a disease, wherein a protease specific to the disease is localized in the cells or tissue comprising the step of contacting the cells with a recombinant protein according to claim 26.

31. A method of treating a disease comprising administering a
20 recombinant protein according to claim 26 to an animal in need thereof.

32. A method of treating a disease comprising administering a nucleic acid molecule according to claim 12 to an animal in need thereof.

33. A method of treating a mammal with cancer or infected with a fungus, virus or parasite, comprising the steps of preparing a recombinant protein of claim 15 wherein the linker sequence contains a cleavage recognition site for a cancer, fungal, viral or parasitic protease and administering the protein to the mammal.

34. A method of inhibiting or destroying cells affected by a disease associated with a protease, comprising contacting cells affected by a disease associated with the protease with a recombinant ricin protein such that cells affected by a disease associated with a protease are inhibited or
5 destroyed, wherein said recombinant ricin protein has an A chain of a ricin-like toxin, B-chain of a ricin-like toxin, and a heterologous linker amino acid sequence containing a cleavage recognition site for the protease associated with the disease.

35. A method according to claim 33, wherein said heterologous linker
10 amino acid sequence contains at least five amino acids which differ from the wild-type.

36. The method of claim 34 where the disease is one of cancer, a fungal infection, or cells infected with a virus or parasite.

37. A process for preparing a pharmaceutical for treating a mammal
15 with cancer, fungal infection, viral infection or parasitic infection, comprising the steps of :

(a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the
20 A and B chains, wherein the linker sequence contains a cleavage recognition site for a cancer, viral or parasitic protease;

(b) introducing the nucleic acid into a host cell and expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino
25 acid sequence;

(c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

38. A pharmaceutical composition for treating cancer or a fungal, or viral, or parasitic infection in an animal comprising the recombinant

protein of claim 16 and a pharmaceutically acceptable carrier, diluent or excipient.

39. A pharmaceutical composition for treating cancer or a fungal, or viral, or parasitic infection in an animal comprising the nucleic acid molecule of claim 3 and a pharmaceutically acceptable carrier, diluent or excipient.
- 5

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0954451 044400

ABSTRACT OF THE DISCLOSURE

The present invention provides a protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence
5 contains a cleavage recognition site for a disease specific protease such as a cancer, fungal, viral or parasitic protease. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cancer cells, cells infected with a virus,
10 a fungus, or parasite, or parasites utilizing the nucleic acid molecules and proteins of the invention and pharmaceutical compositions for treating human cancer, viral infection, fungal infection, or parasitic infection.

093451 04400

Complete Sequence of Baculovirus Transfer Vector, pVL1393

```

ID      PVL1393      preliminary; circular DNA; SYN;
9632 BP.
XX
AC      IG1137;
XX
DT      01-FEB-1993 (Rel. 7, Created)
DT      01-JUL-1995 (Rel. 12, Last updated, Version
1)
XX
DE      E. coli plasmid vector pVL1393 - complete.
XX
KW      cloning vector.
XX
OS      Cloning vector
OC      Artificial sequences; Cloning vehicles.
XX
RN      [1]
RC      p2Bac from baculovirus
RC      p2Blue from p2Bac
RC      pBlueBac from AcNPV
RC      pBlueBac2 from AcNPV
RC      pBlueBacIII from AcNPV
RC      pBlueBacHisA from AcNPV
RC      pBlueBacHisB from AcNPV
RC      pBlueBacHisC from AcNPV
RC      pVL1392, pVL1393 from pAc360
RA      ;
RT      ;
RL      The Digest 5:2-2(1992).
XX
CC      NM (pVL1393)
CC      CM (yes)
CC      NA (ds-DNA)
CC      TP (circular)
CC      ST ()
CC      TY (plasmid)
CC      SP (British
Biotechnology) (Invitrogen)
CC      HO (E.coli NM522) (E.coli
INValphaF') (insect)
CC      CP ()
CC      FN (expression) (transfer)
CC      SE ()
CC      PA (pAC360)
CC      BR (pVL1392)
CC      OF ()
CC      OR ()
XX
FH      Key
FH      Location/Qualifiers

```

FIGURE 1 (Cont'd)

```

FT    misc_feature      0..0
FT                                     /note="1. pAc360, ori/amp/AcMNPV
polyhedrin gene
FT                                     -> pVL1393 9632bp"
FT    transposon        0..0
FT                                     /note="TRN AcMNPV"
FT    misc_binding      868..868
FT                                     /note="SIT SacII"
FT    misc_binding      1395..1395
FT                                     /note="SIT ApaI"
FT    misc_binding      1901..1901
FT                                     /note="SIT XhoI"
FT    promoter          0..0
FT                                     /note="PRO AcMNPV polyhedrin gene"
FT    misc_binding      0..0
FT                                     /note="MCS
FT                                     BamHI-SmaI-XbaI-EcoRI-NotI-XmaIII-PstI-
BglIII"
FT    rep_origin         0..0
FT                                     /note="ORI E. coli pMB1 (ColE1 and
pBR322)"
FT    CDS                complement(0..0)
FT                                     /note="ANT E. coli beta-lactamase gene
(bla)
FT                                     ampicillin resistance gene (apr/amp)"
XX
SQ    Sequence 9632 BP; 2602 A; 2122 C; 2176 G; 2732 T; 0
other;

```

```

aagcttttact cgtaaagcga gttgaaggat catatttagt tgcgtttatg
agataagatt gaaagcacgt gtaaaatggt tcccgcgcggt tggcacaact
atttacaatg cggccaagtt ataaaagatt ctaatctgat atgttttaaa
acacctttgc ggcccgagtt gtttgcgtac gtgactagcg aagaagatgt
gtggaccgca gaacagatag taaaacaaaa ccctagtatt ggagcaataa
tcgattttaac caacacgtct aaatattatg atggtgtgca ttttttgcgg
gcgggcctgt tatacaaaaa aattcaagta cctggccaga ctttgccgcc
tgaaagcata gttcaagaat ttattgacac ggtaaaagaa tttacagaaa
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gcaggaagcc atagatagat tcgaaaaagc cagagggtcac aaaattgaaa
gacaaaatta cgttcaagat ttattaattt aattaatatt atttgcattc
tttaacaaat actttatcct attttcaaat tgttgcgctt cttccagcga
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gttggttccaa tcgacggtag gattaggccg gatattctcc accacaatgt
tggcaacggt gatgttacgt ttatgctttt gggtttccac gtacgtcttt
tggccggtaa tagccgtaaa cgtagtgccg tcgcgcggtca cgcacaacac
cggatgtttg cgcttggtccg cggggtattg aaccgcgcgga tccgacaaat
ccaccacttt ggcaactaaa tcggtgacct gcgcggtcttt tttctgcatt
atttcgtctt tcttttgcac ggtttcctgg aagccggtgt acatgcggtt
tagatcagtc atgacgcgcg tgacctgcaa atctttggcc tcgatctgct
tgtccttgat ggcaacgatg cgttcaataa actcttggtt tttacaagt
tcctcggttt tttgcgccac caccgcttgc agcgcggttg tgtgctcggt
gaatgtcgca atcagcttag tcaccaactg tttgctctcc tctcccgtt
gtttgatcgc gggatcgtag ttgccggtgc agagcacttg aggaattact
tcttctaaaa gccattcttg taattctatg gcgtaaggca atttggactt

```

FIGURE 1 (Cont'd)

cataatcagc	tgaatcacgc	cggatttagt	aatgagcact	gtatgcggct
gcaaatacag	cgggtcgccc	cttttcacga	cgctggtaga	ggtagggccc
ccattttgga	tgggtctgctc	aaataacgat	ttgtatttat	tgtctacatg
aacacgtata	gctttatcac	aaactgtata	ttttaaactg	ttagcgacgt
ccttgccac	gaaccggacc	tgttggtcgc	gctctagcac	gtaccgcagg
ttgaacgtat	cttctccaaa	tttaaattct	ccaattttaa	cgcgagccat
tttgatacac	gtgtgtcgat	tttgcaacaa	ctattgtttt	ttaacgcaaa
ctaaacttat	tgtggttaagc	aataattaaa	tatgggggaa	catgcgcgcg
tacaacactc	gtcgttatga	acgcagacgg	cgccggtctc	ggcgcaagcg
gctaaaacgt	gttgcgcggt	caacgcggca	aacatcgcaa	aagccaatag
tacagttttg	at ttgcatat	taacgcgcat	tttttaaaatt	atcttattta
ataaatagtt	atgacgccta	caactccccg	cccgcgttga	ctcgtgcac
ctcgagcagt	tcgttgacgc	cttcctccgt	gtggccgaac	acgtcgagcg
ggtggtcgat	gaccagcggc	gtgccgcacg	cgacgcacaa	gtatctgtac
accgaatgat	cgtcgggcga	aggcacgtcg	gcctccaagt	ggcaatattg
gcaaattcga	aaatatatac	agttgggttg	tttgcgcata	tctatcgtgg
cgttgggcat	gtacgtccga	acgttgattt	gcatgcaagc	cgaaattaaa
tcattgcat	tagtgcgatt	aaaacgttgt	acatcctcgc	ttttaatcat
gccgtcgatt	aaatcgcgca	atcgagtcaa	gtgatcaaag	tgtggaataa
tgttttcttt	gtattcccga	gtcaagcgca	gcgcgtatatt	taacaaacta
gccatcttgt	aagttagttt	cattttaatgc	aactttatcc	aataatatat
tatgtatcgc	acgtcaagaa	ttaacaatgc	gcccgttgtc	gcatctcaac
acgactatga	tagagatcaa	ataaagcgcg	aattaaatag	cttgcgacgc
aacgtgcacg	atctgtgcac	gcgttcgggc	acgagctttg	attgtaataa
gtttttacga	agcgatgaca	tgacccccgt	agtgacaacg	atcacgcca
aaagaactgc	cgactacaaa	attaccgagt	atgtcggtga	cgttaaaact
attaagccat	ccaatcgacc	gttagtcgaa	tcaggaccgc	tgggtgcgaga
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agagcgtcat	gtttagacaa	gaaagctaca	tattttaattg	atccccgatga
ttttattgat	aaattgaccc	taactccata	cacggtattc	tacaatggcg
gggttttggt	caaaatttcc	ggactgcat	tgtacatgct	gttaacggct
ccgccacta	ttaatgaaat	taaaaattcc	aatttttaaaa	aacgcagcaa
gagaaacatt	tgtatgaaag	aatgcgtaga	aggaaagaaa	aatgtcgtcg
acatgctgaa	caacaagatt	aatatgcctc	cgtgtataaa	aaaaatattg
aacgatttga	aagaaaacaa	tgtaccgcgc	ggcggtatgt	acaggaagag
gtttatacta	aactgttaca	ttgcaaacgt	ggtttcgtgt	gccaaagtgtg
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aaacaattat	aaatgctaaa	tttgtttttt	attaacgata	caaaccaaac
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atcgctgagg	taatatttaa	aatcattttc	aatgatattca	cagttaatatt
gcgacaatat	aattttattt	tcacataaac	tagacgcctt	gtcgtcttct
tcttcgtatt	ccttctcttt	ttcatttttc	tcctcataaa	aattaacata
gttattatcg	tatccatata	tgtatctatc	gtatagagta	aattttttgt
tgtcataaat	atataatgtct	tttttaaatgg	ggtgtatagt	accgctgcgc
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gtgtgttgct	tttaattatta	aatttatata	atcaatgaat	ttgggatcgt
cggttttgta	caatatgttg	ccggcatagt		
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acataacttt	ccaaaatggt	gtacgaaccg	ttaaacaana	acagttcacc
tcccttttct	atactattgt	ctgcgagcag	ttgtttgttg	ttaaaaaata
cagccattgt	aatgagacgc	acaaactaat	atcacaaact	ggaaatgtct

FIGURE 1 (Cont'd)

ctgtcccgat	ttatattgaaa	cactacaaat	taaaggcgag	ctttcgtagc
aacttggttag	caatattatt	agacagctgt	gtgaagcgct	caacgatttg
cacaagcaca	atttcataca	caacgacata	aaactcgaaa	atgtcttata
tttcgaagca	cttgatcgcg	tgtatgtttg	cgattacgga	ttgtgcaaac
acgaaaactc	acttagcggtg	cacgacggca	cgttggagta	ttttagtcgg
gaaaaaattc	gacacacaa	tatgcacgtt	tcgtttgact	ggtacgcggc
gtgttaacat	acaagttgct	aacgtaatat	tgggtcatagc	tgtttcctgt
gtgaaattgt	tatccgctca	caattccaca	caacatacga	gccggaagca
taaagtgtaa	agcctggggg	gcctaattgag	tgagctaact	cacattaatt
gcgttgcgct	cactgcccgc	tttccagtcg	ggaaacctgt	cgtgccagct
gcattaatga	atcggccaa	gcgcggggag	aggcggtttg	cgtattgggc
gctcttccgc	ttcctcgcgc	actgactcgc	tgcgctcggt	cgttcggctg
cggcgagcgg	tatcagctca	ctcaaaggcg	gtaatacggg	tatccacaga
atcaggggat	aacgcaggaa	agaacatgtg	agcaaaaagg	cagcaaaaagg
ccaggaaccg	taaaaaggcc	gcgttgctgg	cgtttttcca	taggctccgc
ccccctgacg	agcatcacaa	aaatcgacgc	tcaagtcaga	ggtggcgaaa
cccgacagga	ctataaagat	accaggcggt	tccccctgga	agctccctcg
tgcgctctcc	tgttccgacc	ctgccgctta	ccggatacct	gtccgccttt
ctcccttcgg	gaagcgtggc	gctttctcat	agctcacgct	gtaggtatct
cagttcgggtg	taggtcgttc	gctccaagct	gggctgtgtg	cacgaacccc
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aacccggtaa				
gacacgactt	atcgccactg	gcagcagcca	ctggtaacag	gatttagcaga
gcgaggtatg	taggcgggtgc	tacagagttc	ttgaagtggg	ggcctaacta
cggctacact	agaaggacag	tattttggtat	ctgcgctctg	ctgaagccag
ttaccttcgg	aaaaagagtt	ggtagctctt	gatccggcaa	acaaaccacc
gctggtagcg	gtggtttttt	tgtttgcaag	cagcagatta	cgcgcagaaa
aaaaggatct	caagaagatc	ctttgatctt	ttctacgggg	tctgacgctc
agtggaaacga	aaactcacgt	taagggattt	tggtcatgag	attatcaaaa
aggatcttca	cctagatcct	tttaaattaa	aatgaagtt	ttaaatcaat
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tgactccccg	tcgtgtagat	aactacgata	cgggaggggc	taccatctgg
ccccagtgtc	gcaatgatac	cgcgagaccc	acgctcaccg	gctccagatt
tatcagcaat	aaaccagcca	gccggaaggg	ccgagcgcag	aagtggctct
gcaactttat	ccgcctccat	ccagctctatt	aattggttgc	gggaagctag
agtaagtagt	tcgccagtta	atagtttgcg	caacgttggt	gccattgcta
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gcaaaaaagg	gaataaggcg	gacacggaaa	tgttgaatac	tcataactct
cctttttcca	tattattgaa	gcatttatca	gggttattgt	ctcatgagcg
gatacatatt	tgaatgtatt	tagaaaaata	aacaaatagg	ggttccgcgc
acatttcccc	gaaaagtgcc	acctgacgtc	taagaaacca	ttattatcat
gacattaacc	tataaaaata	ggcgtatcac	gaggcccttt	cgtctcgcgc
gtttcgggtga	tgacggtgaa	aacctctgac	acatgcagct	cccgagagacg
gtcacagctt	gtctgtaagc	ggatgccggg	agcagacaag	cccgctcaggg

FIGURE 1 (Cont'd)

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atcaatatat agttgctgat atcatggaga taattaaaat gataaccatc
tcgcaaataa ataagtattt tactgttttc gtaacagttt tgtaataaaa
aaacctataa atattccgga ttattcatat cgtcccacca tcgggcgcg
atcccgggta ccttctagaa ttccggagcg gccgctgcag atctgatcct
ttcctgggac ccggcaagaa ccaaaaactc actctcttca aggaaatccg
taatgttaaa cccgacacga tgaagcttgt cgttggtatg aaaggaaaag
agttctacag ggaaacttgg acccgcttca tggaagacag cttccccatt
gttaacgacc aagaagtgat ggatgttttc cttgttgtea acatgcgtcc
cactagaccc aaccgttgtt acaaattcct ggcccaacac gctctgcgtt
gcgaccccgga ctatgtacct catgacgtga ttaggatcgt cgagccttca
tgggtgggca gcaacaacga gtaccgcac agcctggcta agaaggcg
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atcggtagcg actctgctga agaggaggaa attctccttg aagtttccct
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gattctgtgc gttgttgatt tacagacaat tgttgtacgt attttaataa
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gattttcagc gtctttatat ctgaatttaa atattaaatc ctcaatagat
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tgtaataaag gttcgacgtc gttcaaaaata ttatgcgctt ttgtatttct
ttcatcactg tcgttagtgt acaattgact cgacgtaaac acgttaaata
aagcttgga atatttaaca tcgggcgtgt tagctttatt aggccgatta
tcgtcgtcgt cccaaccctc gtcgttagaa gttgcttccg aagacgattt
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tgggcgggtt tcaatctaac tgtgccgat ttttaattcag acaacacgtt
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atggcggcgg tgggtggagc gatgataaat ctaccatcgg tggaggcgca
ggcggggctg ggcggcgagg cggaggcgga ggtggtggcg gtgatgcaga
cggcggttta ggctcaaatg tctctttagg caacacagtc ggcacctcaa
ctattgtact ggtttcgggc gccgtttttg gtttgaccgg tctgagacga
gtgcgatttt tttcgtttct aatagcttcc aacaattggt gtctgtcgtc
taaaggtgca gcgggttgag gttccgtcgg cattggtgga gcgggaggca
attcagacat cgatgggtgg ggtggtggcg gaggcgctgg aatggtaggc
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agtttggtcg cgcacgattg tgggcaccgg cgcaggcgcc gctggctgca
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ttttcatttt tactacagca ttgtagtggc gagacacttc gctgtcgtcg
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gcatcaattt tgttgttcc attattgaat aaataagatt gtacagattc
atatctacga ttcgtcatgg ccaccacaaa tgctacgctg caaacgctgg
tacaatttta cgaaaactgc aaaaacgtca aaactcggt taaaataatc
aacgggcgct ttggcaaaat atctatttta tcgcacaagc ccactagcaa
attgtatttg cagaaaacaa tttcggcgca caattttaac gctgacgaaa
taaaagttca ccagttaatg agcgaccacc caaattttat aaaaatctat
tttaatcacg gttccatcaa caaccaagt atcgtgatgg actacattga

```

FIGURE 1 (Cont'd)

cgcgtcagcg ggtggtggcg ggtgtcgggg ctggcttaac tatgcggcat
cagagcagat tgtactgaga gtgcaccata tgcggtgtga aataccgcac
agatgcgtaa ggagaaaata ccgcatcagg cgccattcgc cattcaggct
gcgcaactgt tgggaagggc gatcggtgcg ggcctcttcg ctattacgcc
agctggcgaa agggggatgt gctgcaaggc gattaagttg ggtaacgcca
gggttttccc agtcacgacg ttgtaaaacg acggccagtg cc

//

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7/254

FIGURE 2A

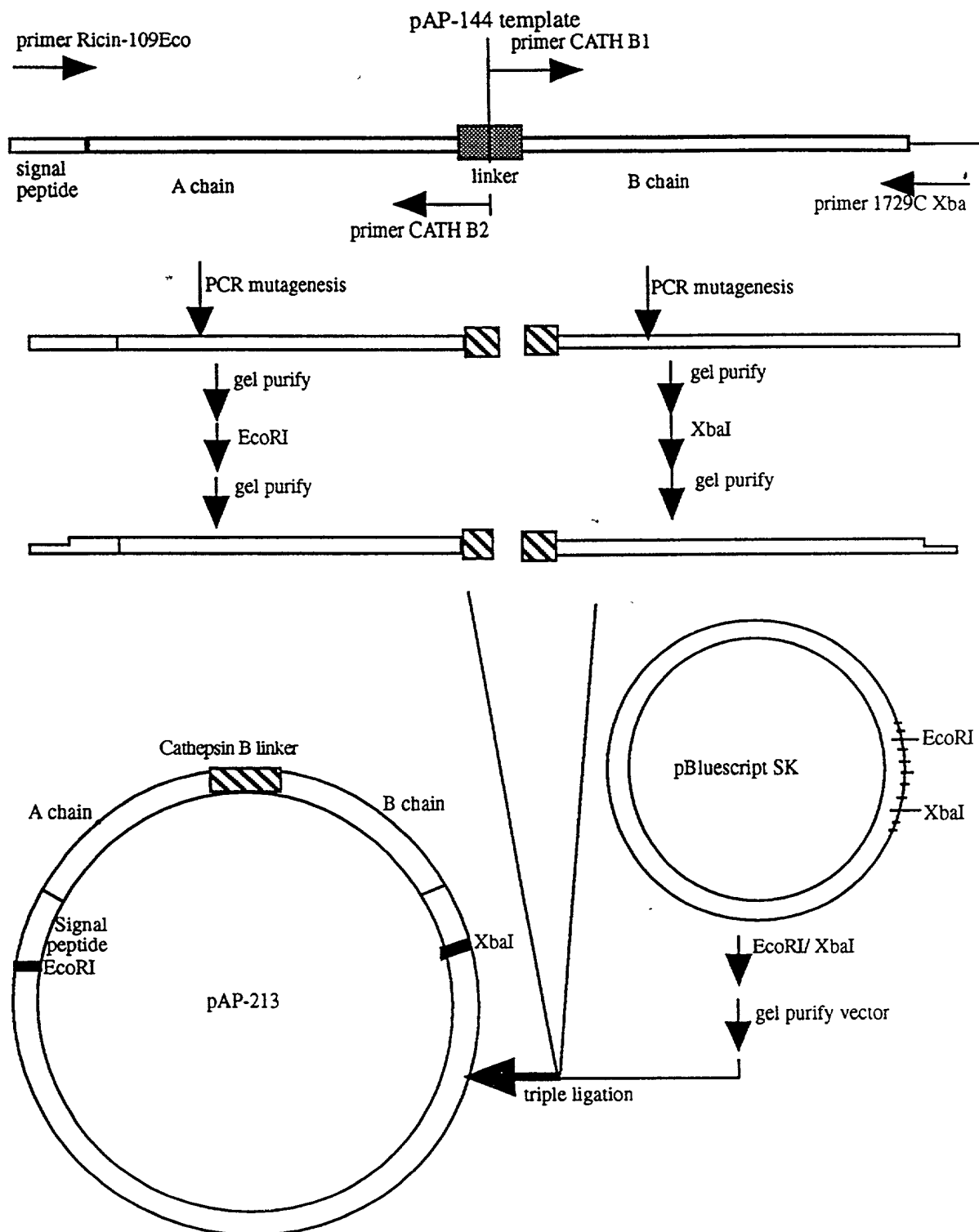


FIGURE 2B

WT preproricin linker

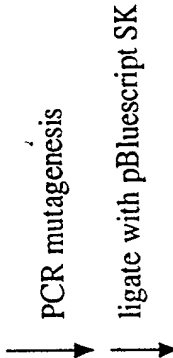
primer CATH-B1

5' - ATGGTGCCAAATTTTAAT-3'

———— TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT ————
———— AGAAACGAATATTCCGGTCAACACGGTTAAAATTA ————

3' - TCTCGATTTAAGCAAGAAACTG-5'

primer CATH-B2

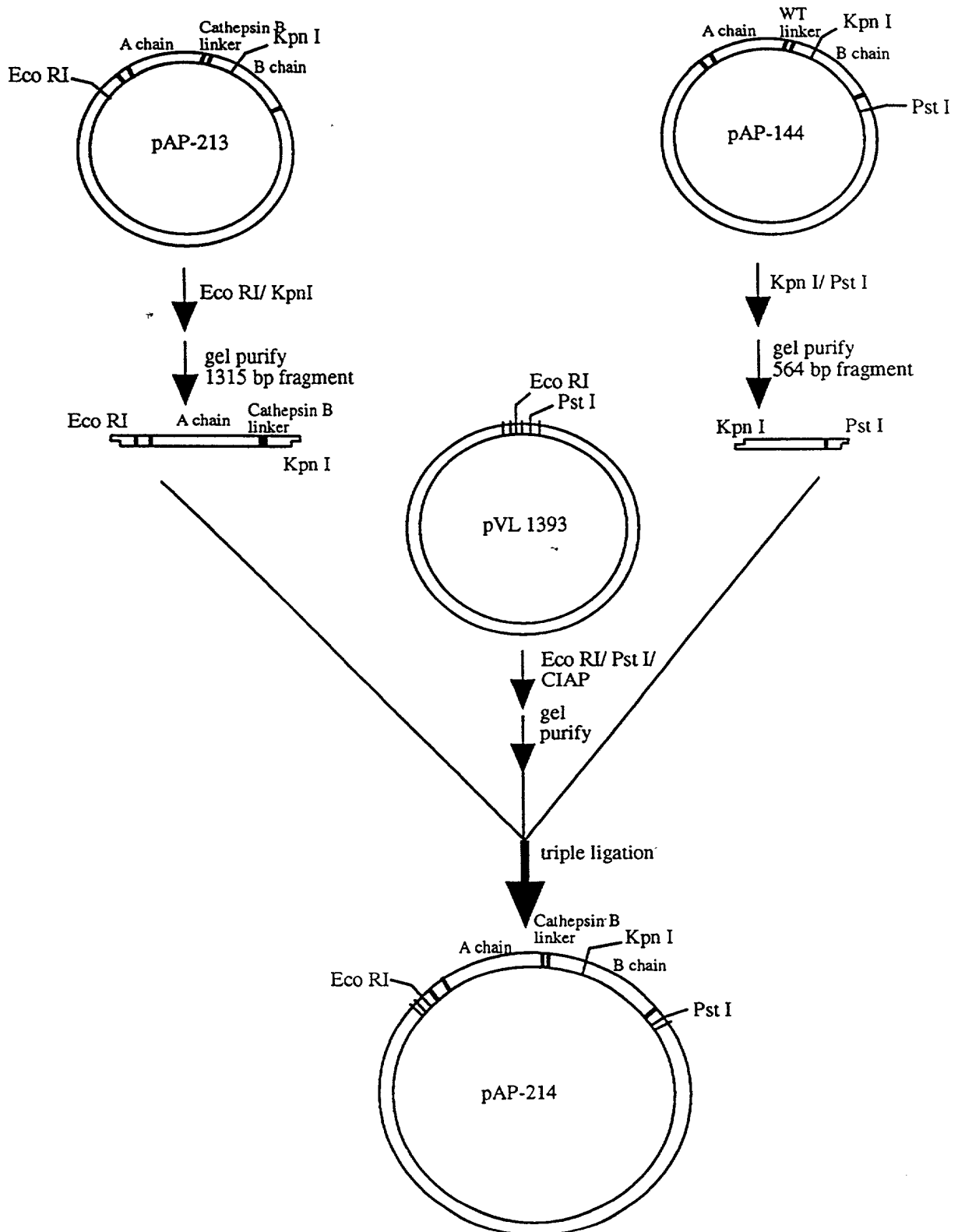


**pAP 213 linker
(Cathepsin-B variant)**

———— TCTTTGCTTAAATCGAGAAATGGTGCCAAATTTTAAT ————
———— AGAAACGAATTTAGCTCTTACCACGGTTAAAATTA ————

9/254

FIGURE 2C



10/254

FIGURE 2D

10 20 30 40 50

1 GAATTCATGAAACCGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAACT

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTGTTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAAC TGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACCTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGA CTACAAGTTTTA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC T
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT

901 TCGTCACAGTTTTCTTTTGCTTAAATCGAGAATGGTGCCAAATTTTAATGC
AGCAGTGTCAAAAGAAACGAATTTAGCTCTTACCACGGTTTAAAATTACG

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FIGURE 2D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTACGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCTTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCCTCAG
 TCCGACTTGTGTACCCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
 AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTCTCTGCCATGAAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGTACTTTTATCTACCGAATTTATTTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

12/254

FIGURE 3A

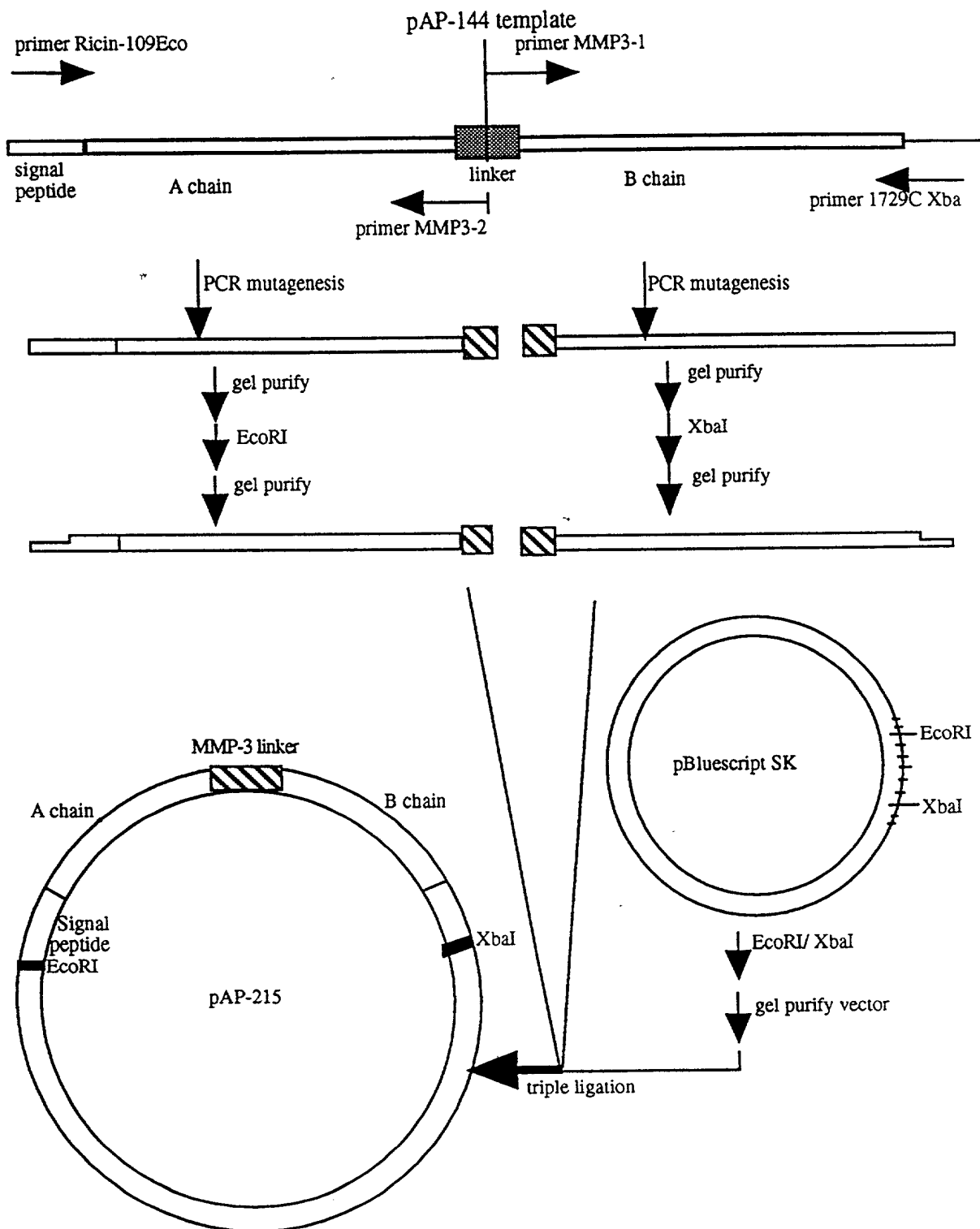


FIGURE 3B

WT preprorin linker

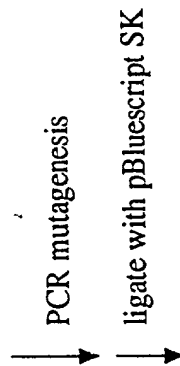
primer MMP3-1

5' - TTTTGGACTTATGAATGCTGATGTTTGT -3'

TTTTCGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCGGTCACCCACGGTTTAAATTA

3' - GGTAGCAGTGTCAAAGCAGGCTTCGGTTCGTT -5'

primer MMP3-2



pAP 215 linker
(MMP-3 variant)

CGTCCGAAGCCACAGCAATTTTGGACTTATGAAT
GCAGGCTTCGGTTCGTTAAAAAACCTGAATACTTA

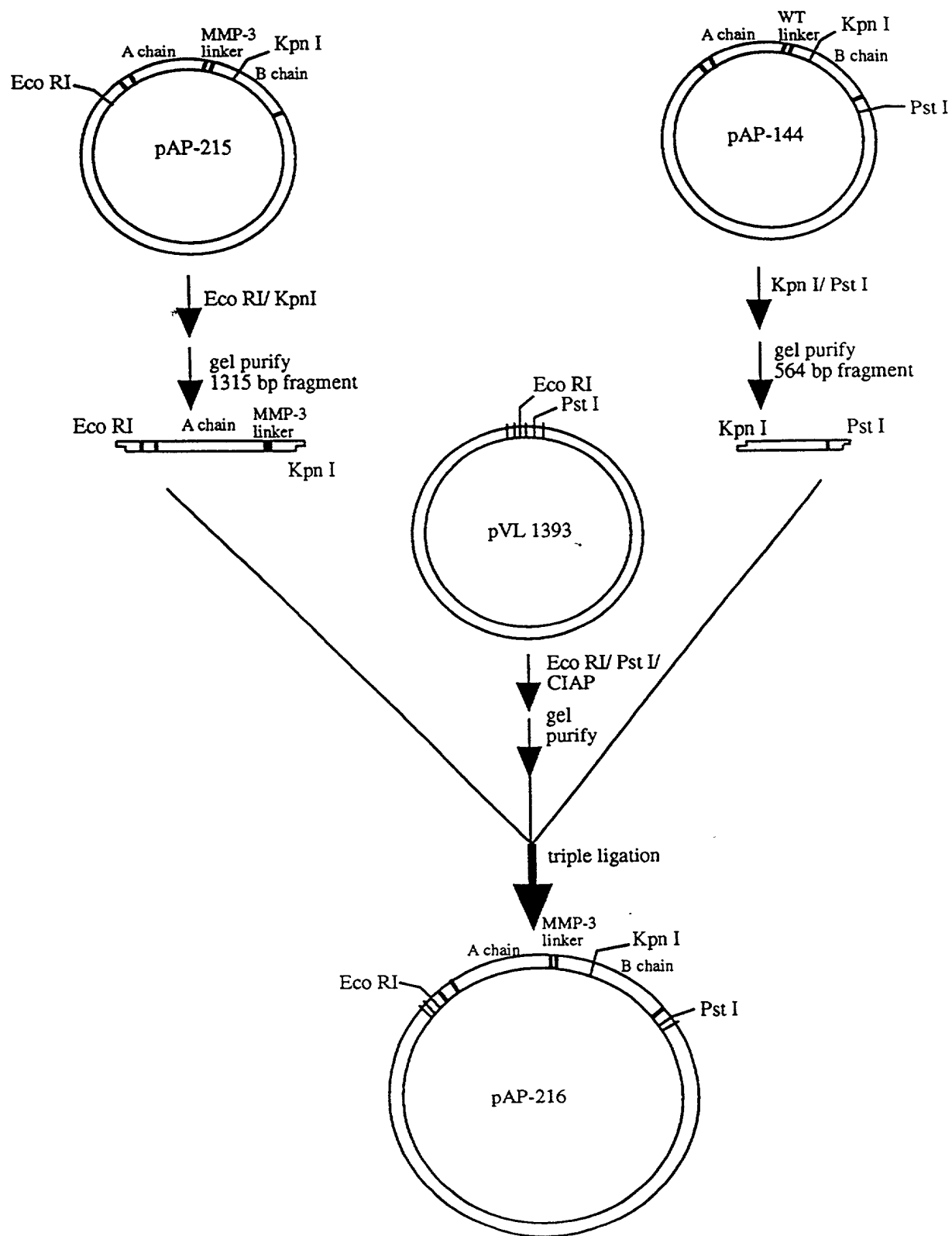
FIGURE 3C

FIGURE 3D

10 20 30 40 50
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTTCGCGG
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCGCA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAATAGCGCATATTTCTTTCATCCTGACA
 ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
 GCTATATGTAAGCGGAAACCACCATTAACTACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
 AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTTGGT
 901 TCGTCACAGTTTCGTCCGAAGCCACAGCAATTTTTTGGACTTATGAATGC
 AGCAGTGTCAAAGCAGGCTTCGGTGTCTGTTAAAAAACCTGAATACTTACG
 951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCGAAATG

FIGURE 3D (CONT'D)

ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTGCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCCTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

FIGURE 4A

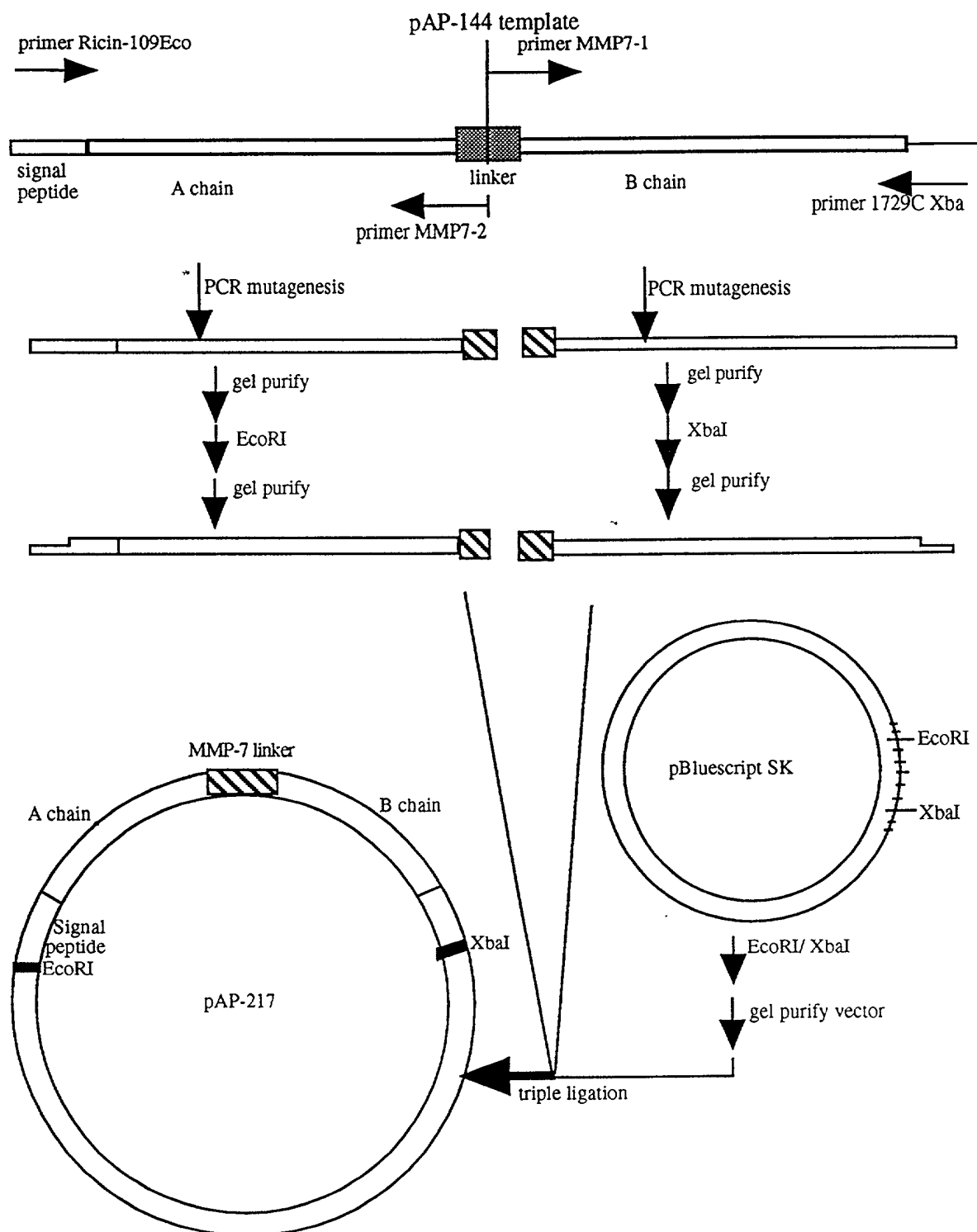


FIGURE 4B

WT preproricin linker

primer MMP7-1

5' - TTGTGGCGAAGTTTAAATGCTGATGTT-3'

TCCTTGCTTATAAGGCCAGTGGTGCCCAATTTTAAT
AGAAACGAAATATCCGGTCACCCACGGTTAAATTA

3' - AGTGTCAAAAGAACGAGGTGACCGT-5'

primer MMP7-2

PCR mutagenesis

ligate with pBluescript SK

pAP 217 linker
(MMP-7 variant)

TCCTTGCGTCCACTGGCATTTGTGGCGAAGTTTAAAT
AGAAACGCAGGTGACCGTAACACCGCTTCAAAATTA

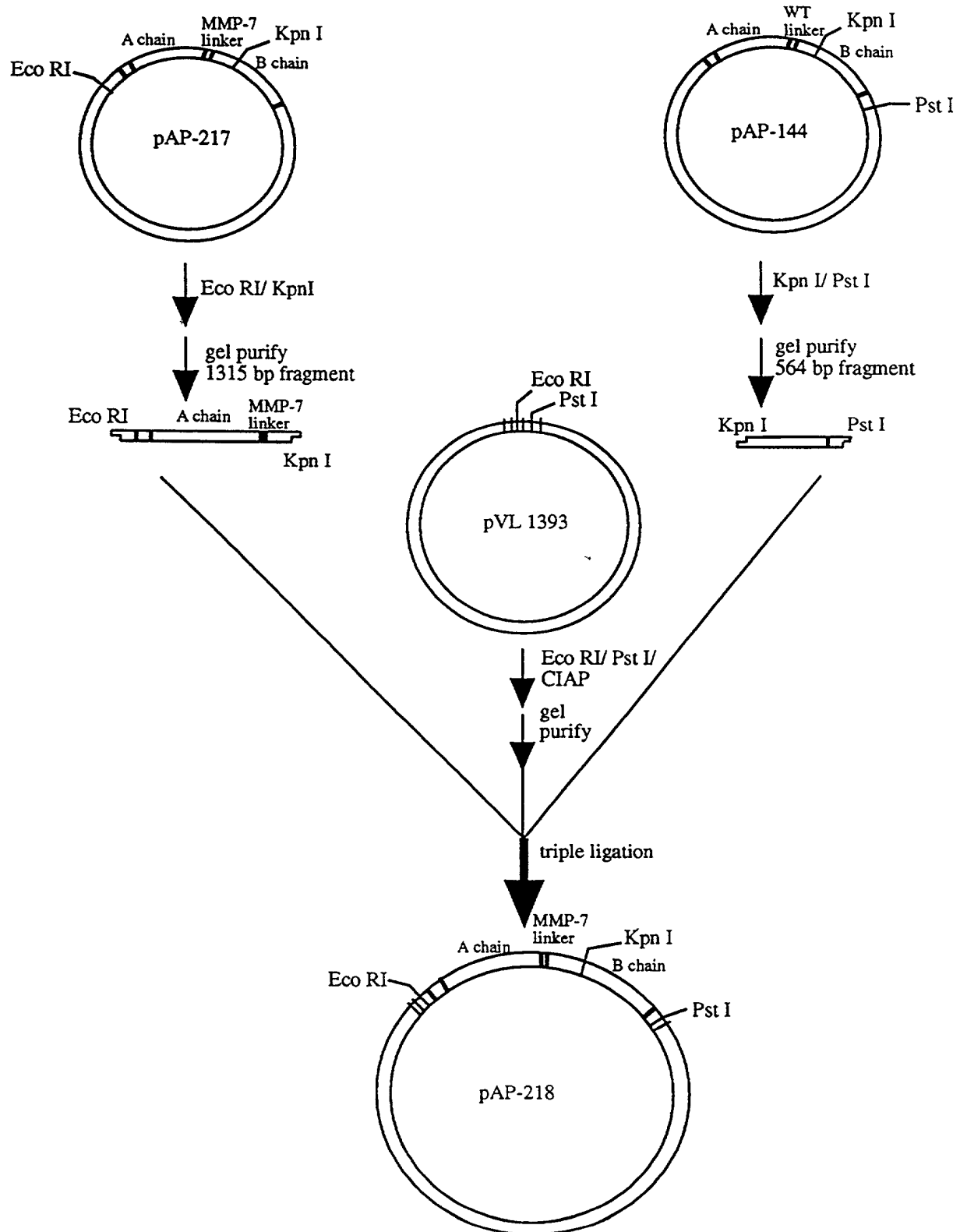
FIGURE 4C

FIGURE 4D

10 20 30 40 50
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
 TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTGTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA
 451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
 AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCAACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT
 901 TCGTCACAGTTTTCTTTGCGTCCACTGGCATTGTGGCGAAGTTTTAATGC
 AGCAGTGTCAAAGAAACGCAGGTGACCGTAACACCGCTTCAAATACG
 951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATATCGTAGGTGAAATG

FIGURE 4D (CONT'D)

ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

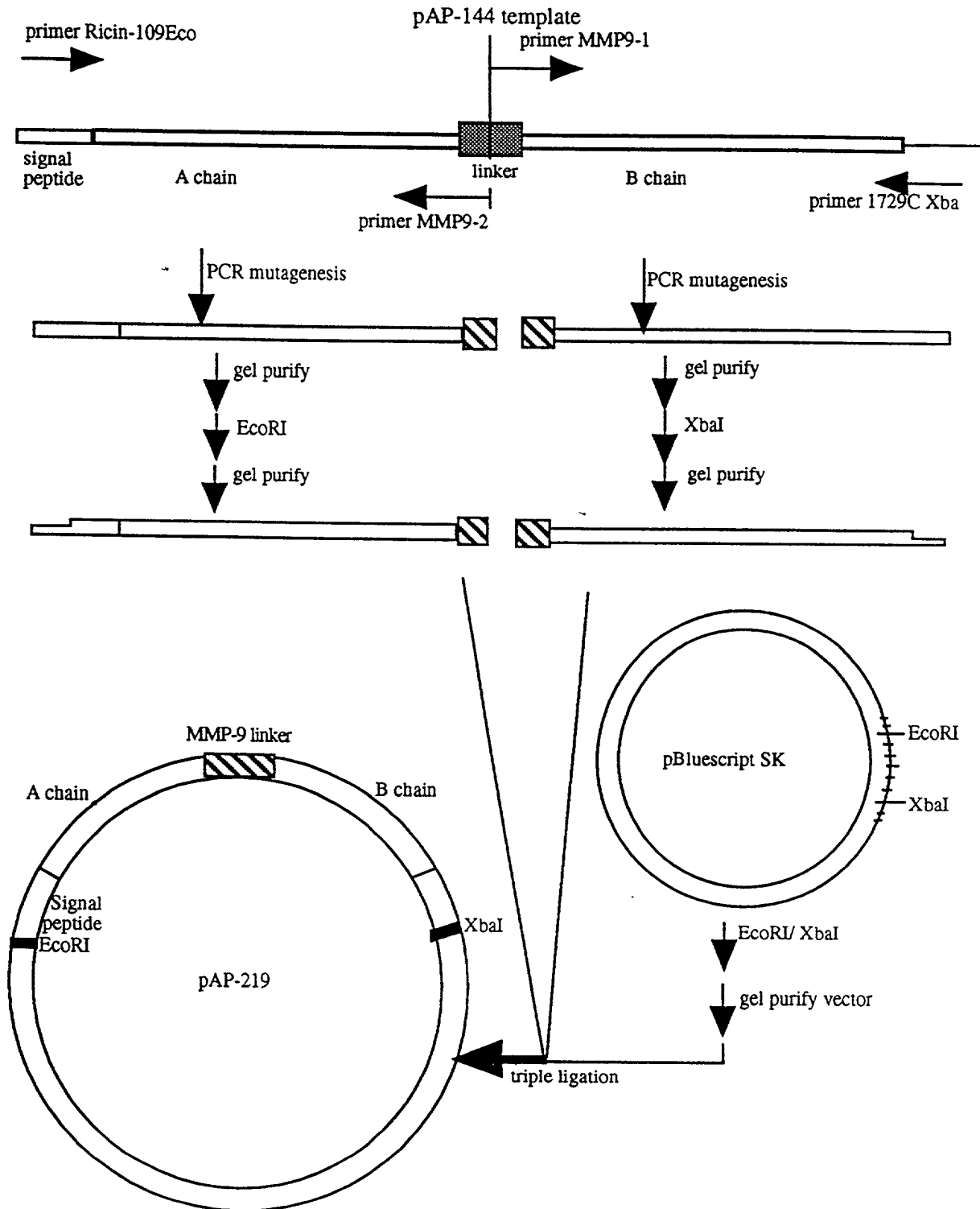
1751 CTCTTGCAGTGTGTGTGCTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTTCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

22/254

FIGURE 5A

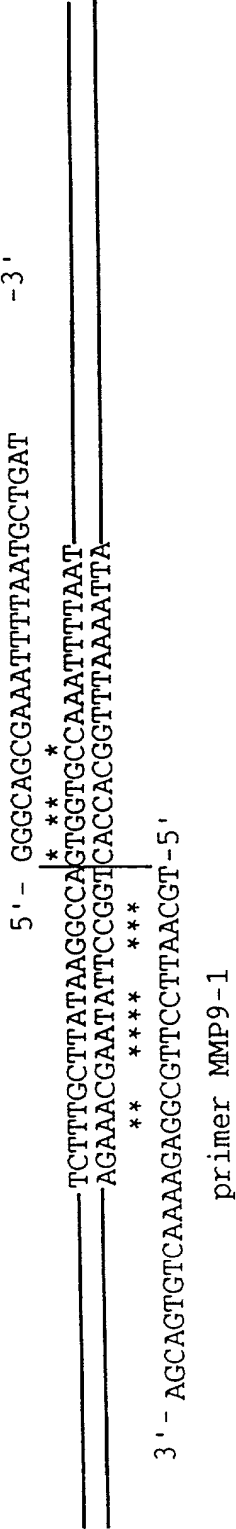


004440" T5T5560

FIGURE 5B

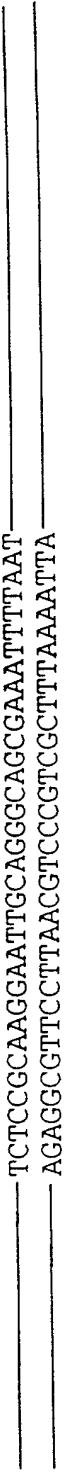
WT preprorenin linker

primer MMP9-1



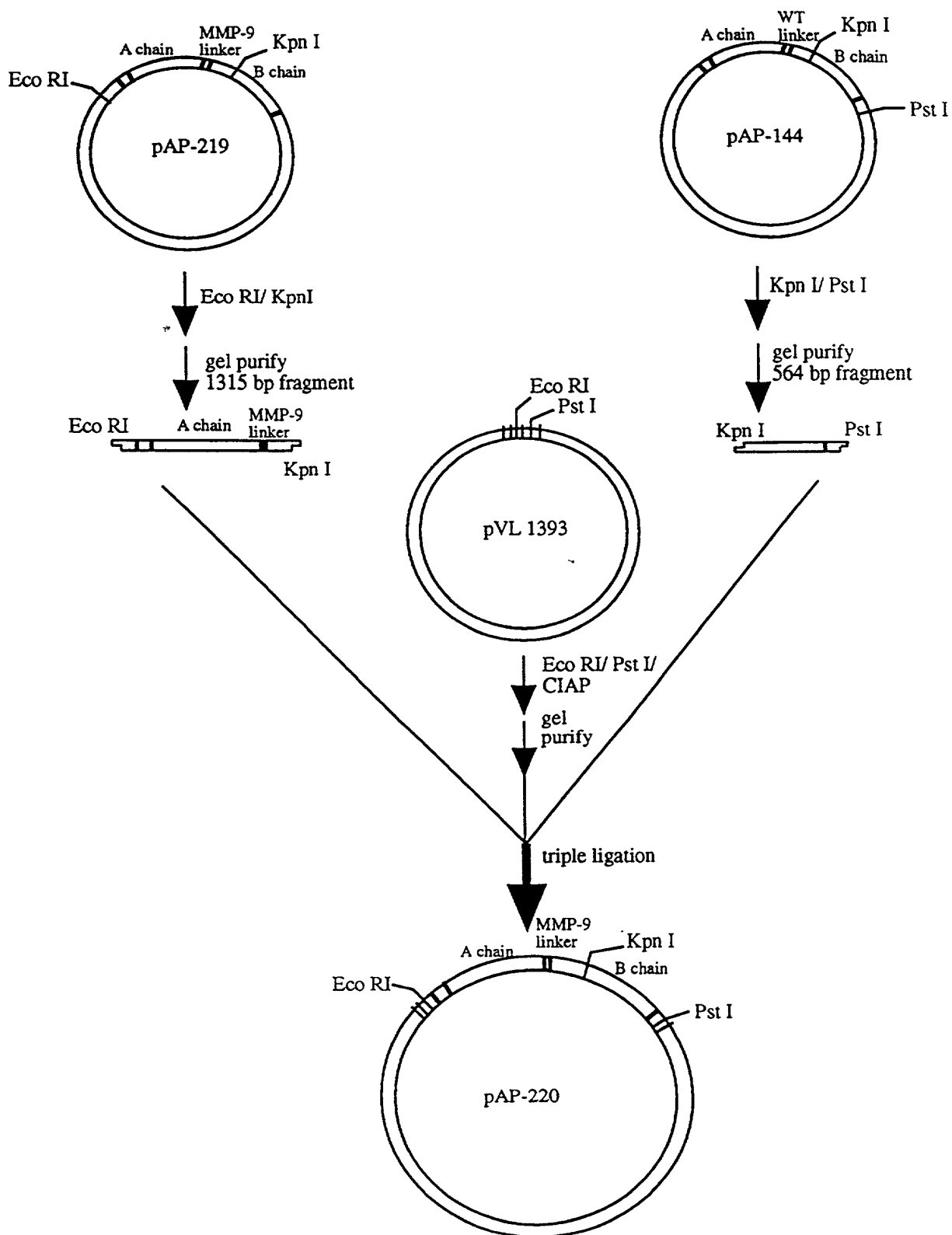
PCR mutagenesis
ligate with pBluescript SK

pAP 219 linker
(MMP-9 variant)



24/254

FIGURE 5C



00440" T9T9560

FIGURE 5D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAAGCTACACAAACTTTATCAGAGCTGTTGCGCG
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCAGCACCCTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTC

451 CGATATACATTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCCTTTATAATTGTCATCCAAATGATTTTCAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTTC

651 ATTCCAATATATTGAGGGAGAAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTCCGCAAGGAATTGCAGGGCAGCGAAATTTTAATGC
AGCAGTGTCAAAAGAGGCGTTCCCTTAACGTCCCGTCGCTTTAAATATACG

FIGURE 5D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAAACATTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAAGCTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAAG
 ACGTC

FIGURE 6A

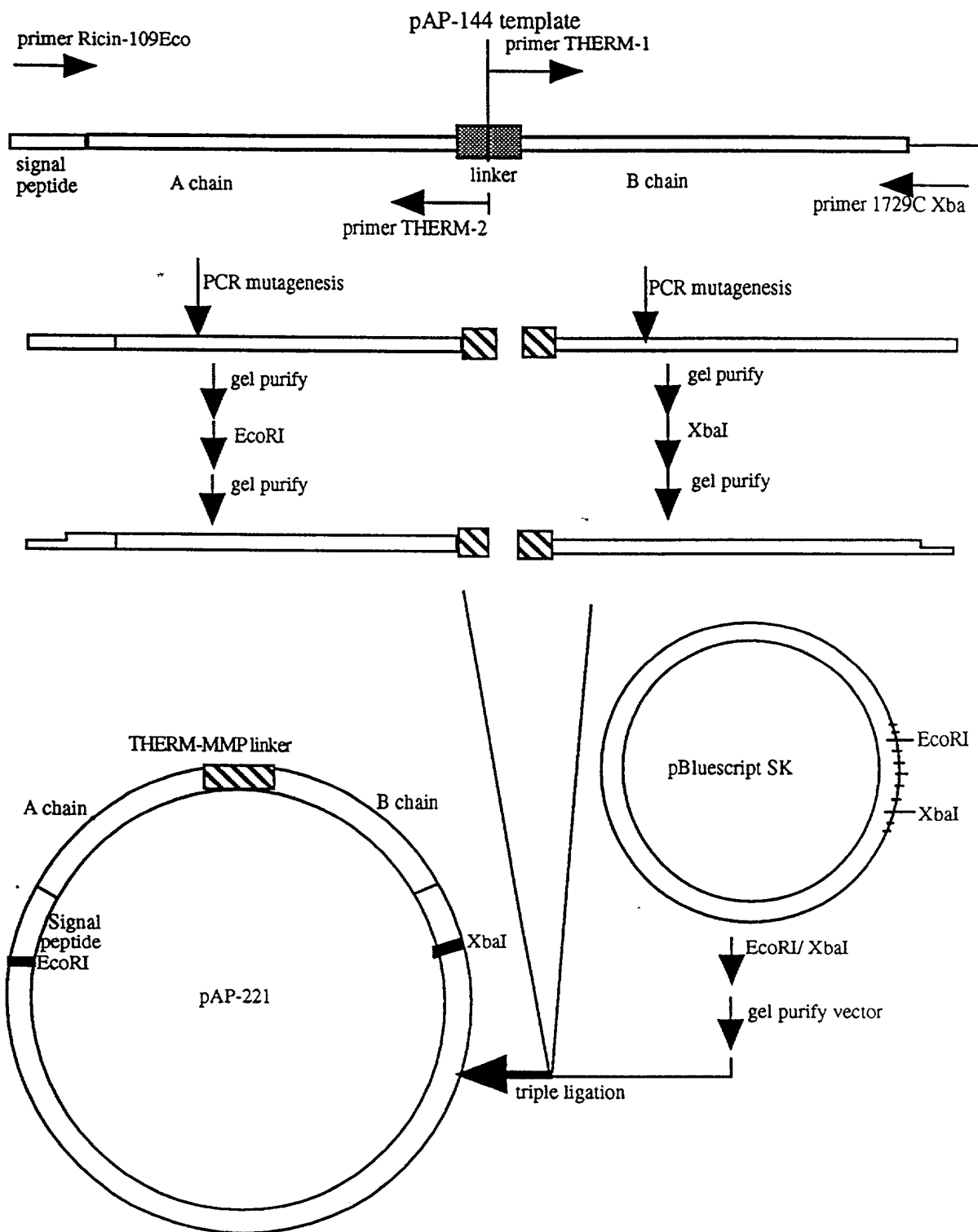


FIGURE 6B

WT preprorin linker

primer THERM-1

5'- AGGGAATTGCTTCTTTTATGCTGATGTTGTATG -3'

TCCTTGCTTATAAGGCCAGTGCTGCCAAATTTAAT
AGAAACGAATATTCCGGTCACACGGTTAAATTA

GGTGGTAGCAGTGTCAAACTACACCTACTTCCCTACAC -5'

3'-

primer THERM-2

PCR mutagenesis
ligate with pBluescript SK

pAP 221 linker
(THERM-MMP variant)
New Cleavage Site

GATGTGGATGAAAGGGATGTGAGGGAATTGCTTCTTTTA
CTACACCTACTTCCCTACACTCCCTTAAACGAAGAAAAAT

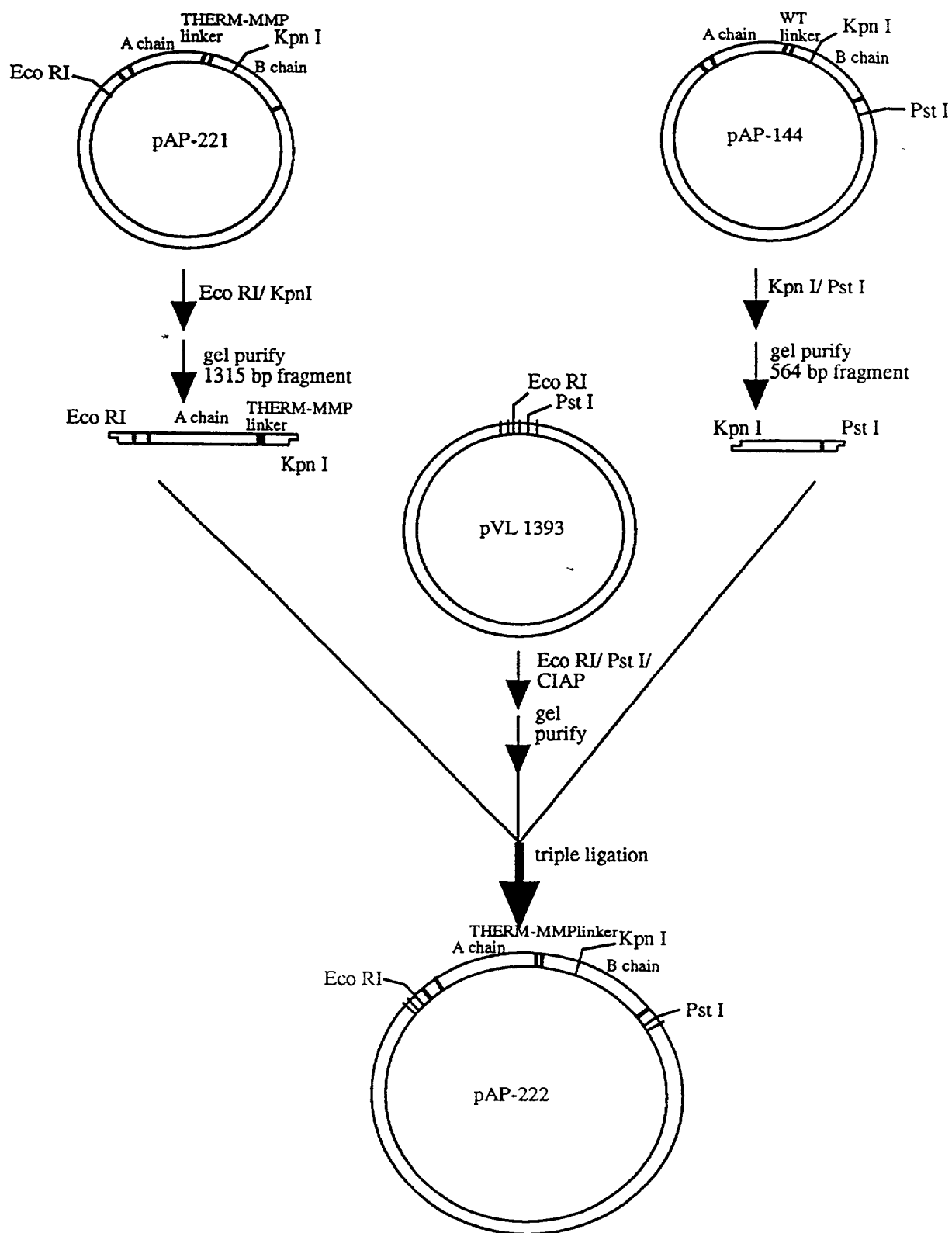
FIGURE 6C

FIGURE 6D

10 20 30 40 50
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA
 ACGCAAATGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTTGCCTATAAACCACGGTTTATTTTAGTTGAACCTCTCA
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
 ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
 451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
 AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTTGATGTGGATGAAAGGGATGTGAGGGAATTTGCTTCTTT
 AGCAGTGTCAAACCTACACCTACTTCCCTACACTCCCTTAAACGAAGAAA
 951 TTTAGCTGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTC

FIGURE 6D (CONT'D)

AAATCGACTACAAACATACC TAGGACTCGGGTATCACGCATAGCATCCAG

1001 GAAATGGTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAAC
CTTTACCAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTG

1051 GCAATACAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTG
CGTTATGTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGAC

1101 GACTTTGAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTA
CTGAAACTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGAT

1151 CTTACGGGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACT
GAATGCCCATGTGAGGCCCTCAGATACACTACTAGATACTAACGTTATGA

1201 GCTGCAACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCAT
CGACGTTGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGGTAGTA

1251 AAATCCCAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGA
TTTAGGGTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCAT

1301 CCACACTTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTT
GGTGTGAATGTACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAA

1351 CCTACTAATAATACACAACCTTTTGTGTTACAACCATTGTTGGGCTATATGG
GGATGATTATTATGTGTTGGAAAACAATGTTGGTAAACAACCCGATATACC

1401 TCTGTGCTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCA
AGACACGAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGT

1451 GTGAAAAGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGT
CACTTTTCCGACTTGTTGTACCCGAGAAATACGTCTACCAAGTTATGCA

1501 CCTCAGCAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGA
GGAGTCGTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCT

1551 AACAGTTGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGAT
TTGTCAACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTA

1601 GGATGTTCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTG
CCTACAAGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAC

1651 TTAGATGTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCC
AATCTACACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGG

1701 TCTCCATGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAG
AGAGGTACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTC

1751 ATTACTCTCTTGCAAGTGTGTGTCTGCCATGAAAATAGATGGCTTAA
TAATGAGAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTT

1801 TAAAAAGGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCG
ATTTTTCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGC

1851 AATTCCTGCAG
TTAAGGACGTC

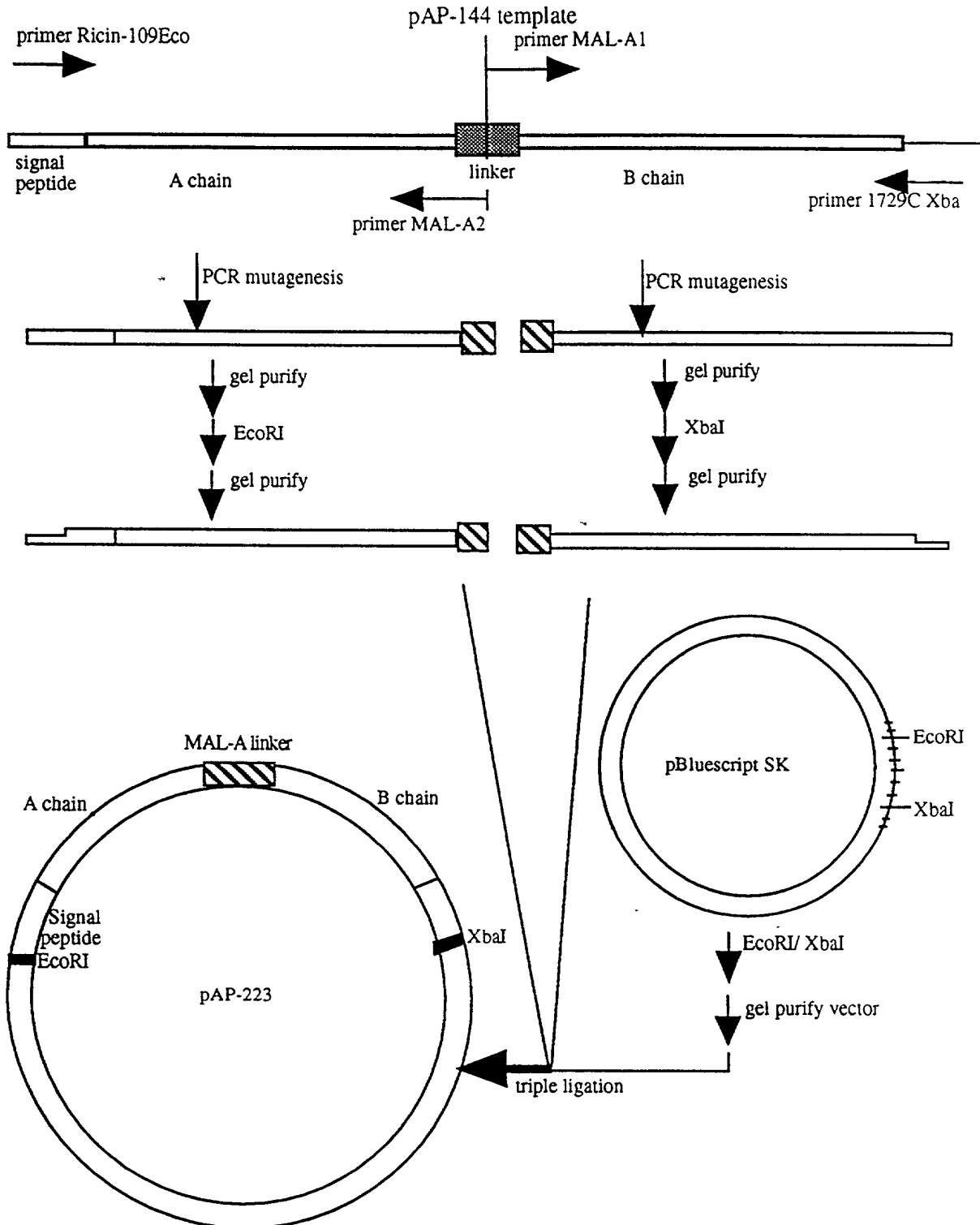
FIGURE 7A

FIGURE 7B

WT preproricin linker

primer MAL-A1

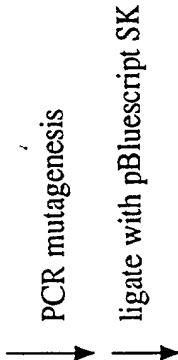
5' - AATTATGATGAAGAGGATGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT

AGAAACGAATATTCCTGGTTCACCAACGGTTTAAATA

3' - GGTAGCAGTGTCAAAGTCCCAAGTTAACGTC -5'

primer MAL-A2



pAP 223 linker
(MAL-A variant)

CAGGTGGTTCAATTGCAGAATTATGATGAAGAGGAT

GTCCACCAAGTTAAGTCTTAATACTACTTCTCCTA

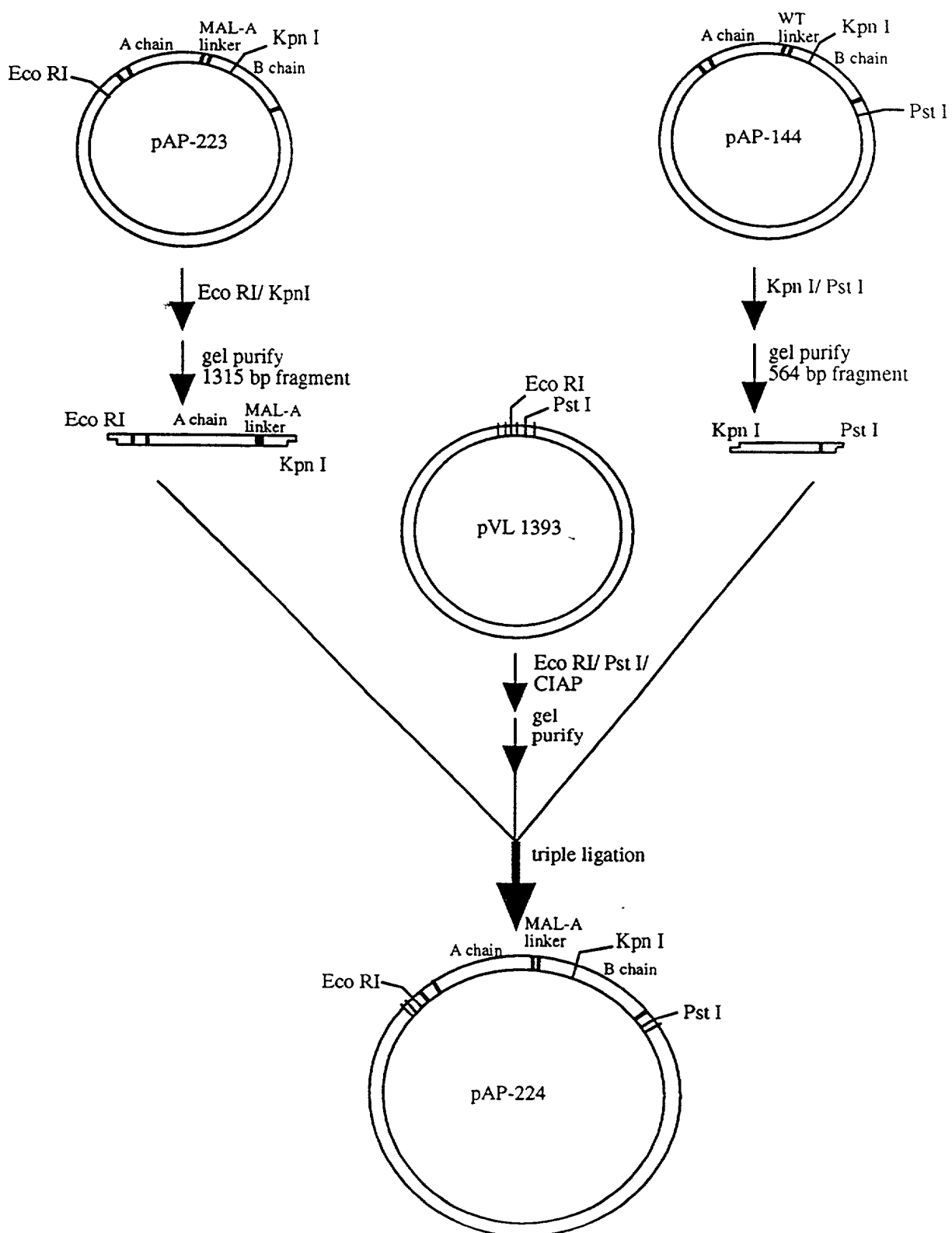
FIGURE 7C

FIGURE 7D

10 20 30 40 50
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTTCGTGTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
 ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
 GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCCTTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
 AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTTTCAGGTGGTTCAATTGCAGAATTATGATGAAGAGGATGC
 AGCAGTGTCAAAGTCCACCAAGTTAACGTCTTAATACTACTTCTCCTACG

FIGURE 7D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAAATCAGCTCTGGACTTT
 GTCAACACCCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTACAAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGAATACGTTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTTCACAACCATTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTTGTCAACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
 AGTTCCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAACATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

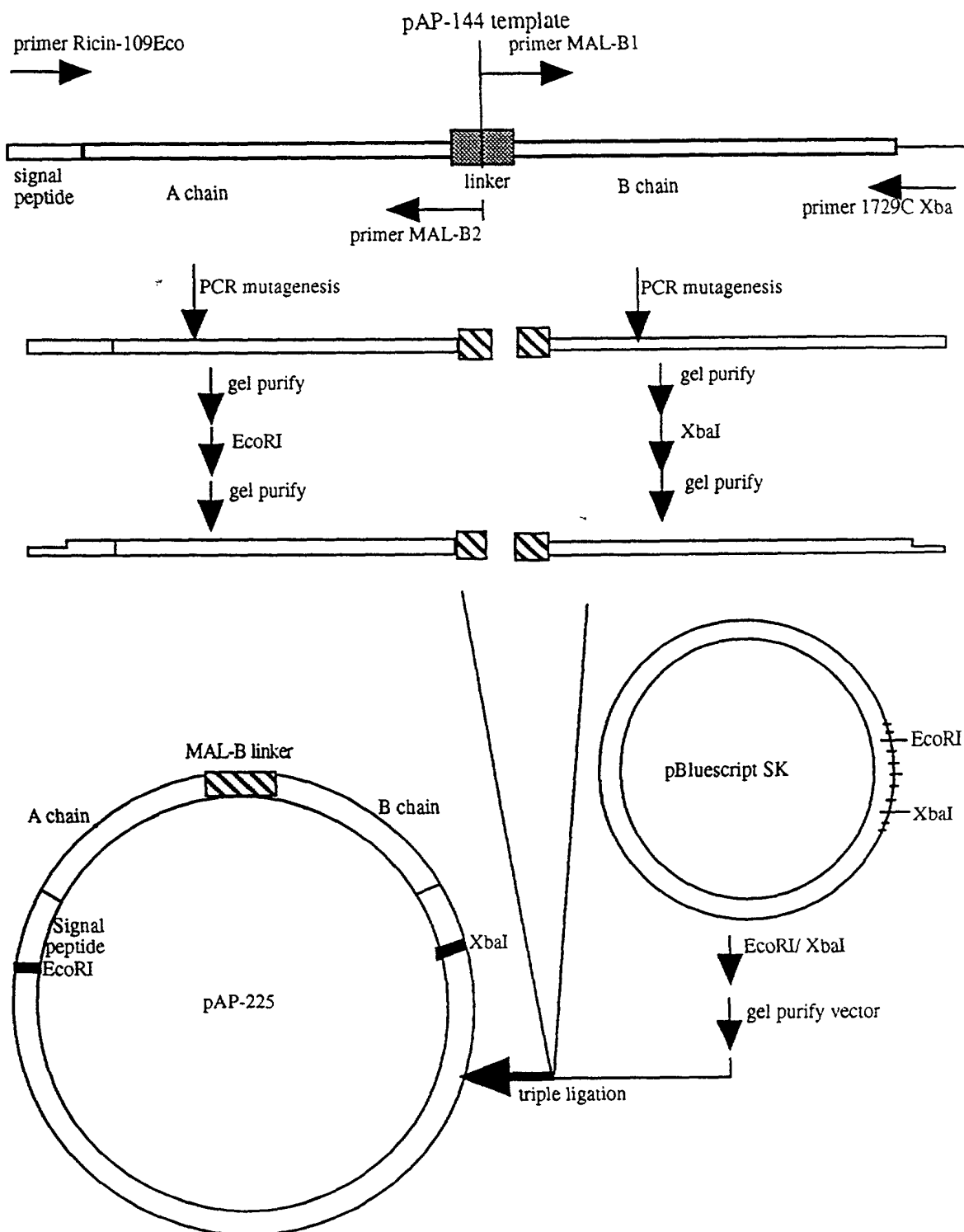
FIGURE 8A

FIGURE 8B

WT preprorin linker

primer MAL-B1

5' - TCGGAGGACAATGATGAAGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAAATATTCCGGTCAACACGGTTTAAAAATTA

3' - GGTAGCAGTGTCAAAAACGGCTAAAAGCCCCCTT -5'

primer MAL-B2

PCR mutagenesis

ligate with pBluescript SK

pAP 225 linker
(MAL-B variant)

TTGCCGATTTTCGGGGAATCGGAGGACAATGATGAA
AACGGCTAAAAGCCCCCTTAGCCTCCTGTACTACTT

39/254

FIGURE 8C

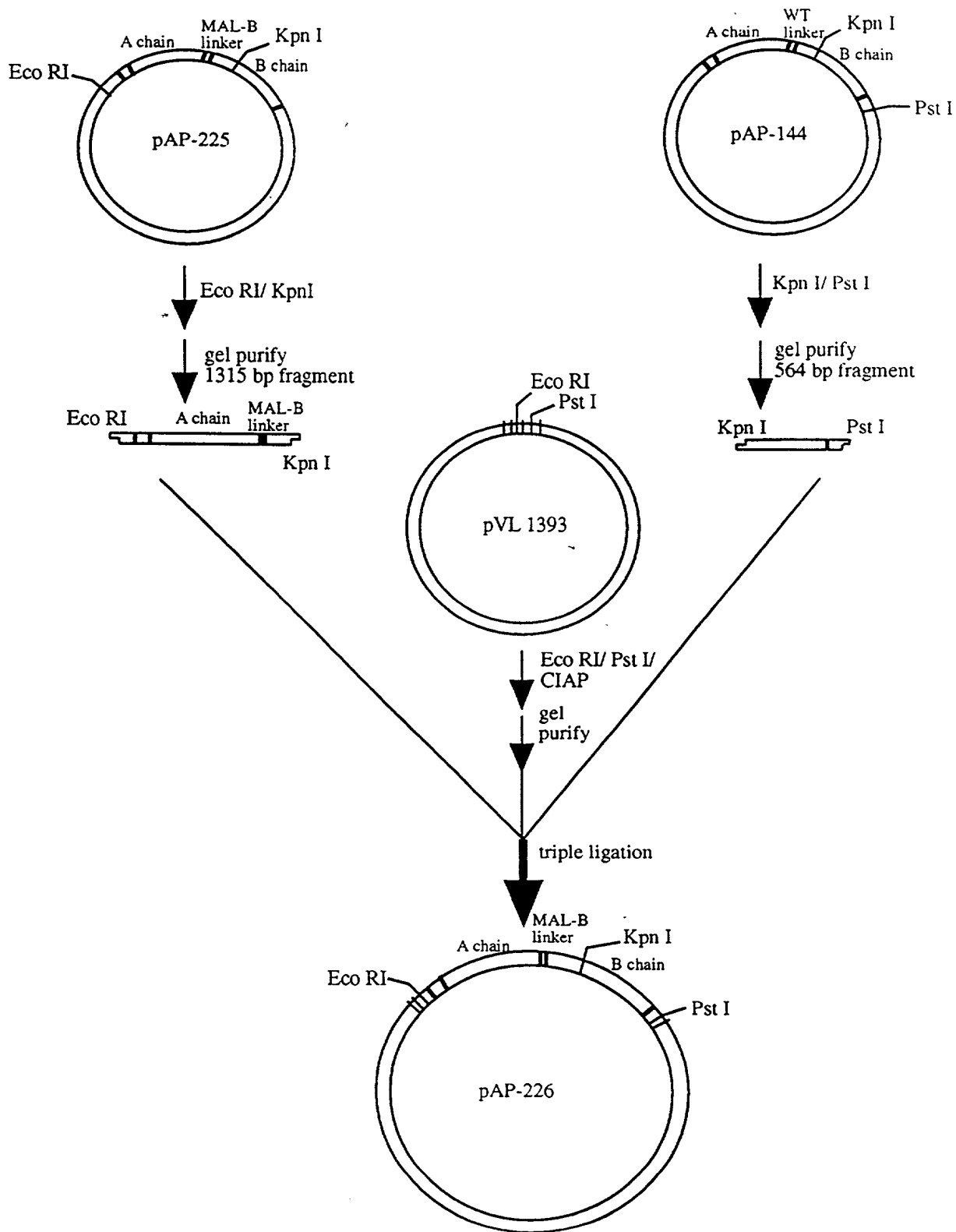


FIGURE 8D

10 20 30 40 50
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTACATTAG
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTGTTATGGGTAAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
 CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTTAACTGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGTGGAATAGCGCATATTTCTTTCATCCTGACA
 ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
 TAGTCCCTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
 451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTTCCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
 AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTTTTGCCGATTTTCGGGGAATCGGAGGACAATGATGAAGC
 AGCAGTGTCAAAAACGGCTAAAAGCCCTTAGCCTCCTGTTACTACTTCG

FIGURE 8D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGC GTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTT CAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCAACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGTAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

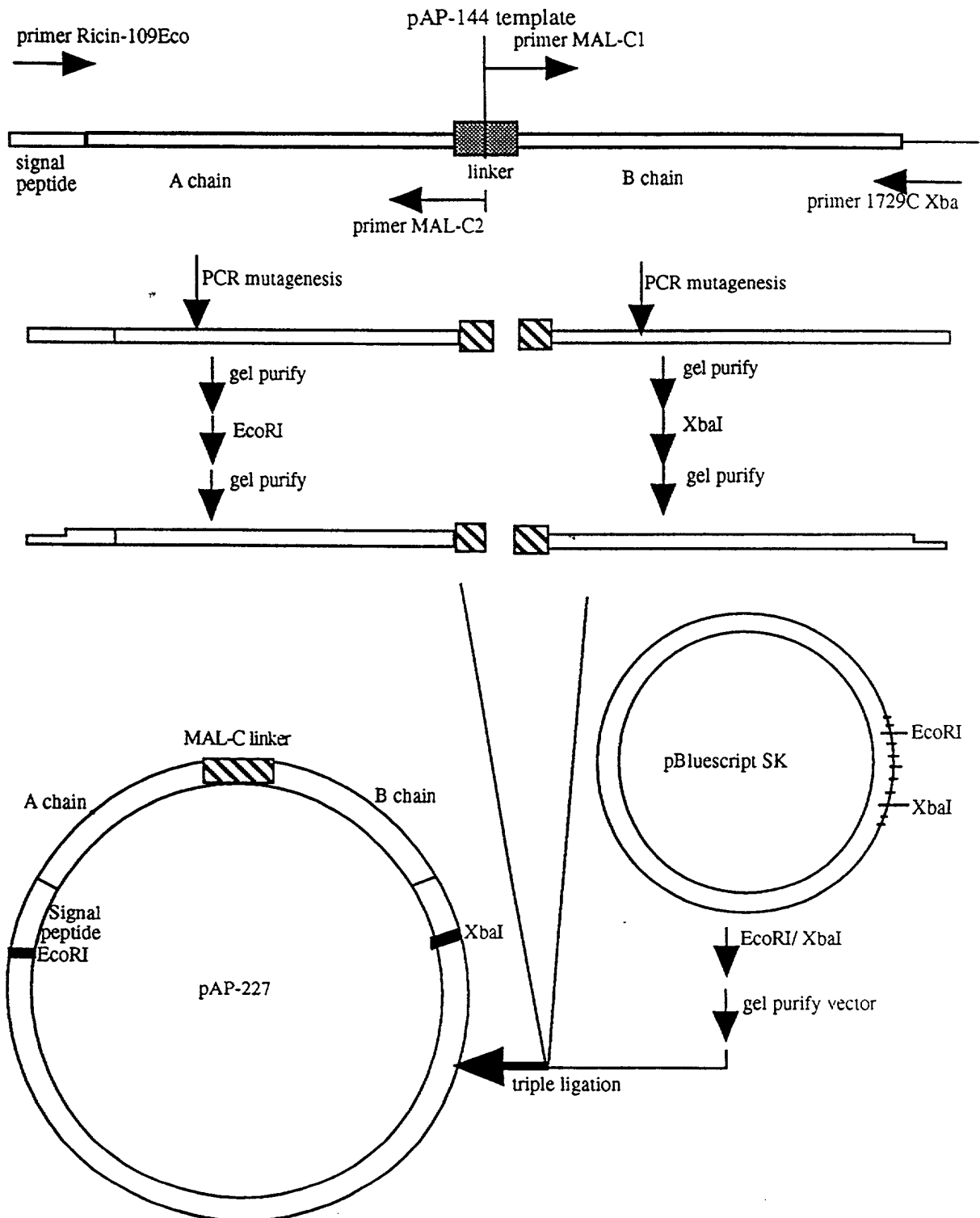
FIGURE 9A

FIGURE 9B

WT preprorin linker

primer MAL-C1

5' - GCGATATCAGTTACTATGGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCTGGTCAACACGGTTTAAATTA

3' - GGTAGCAGTGTCAAAGTCCACCAATGTCCCCCTT -5'

primer MAL-C2

PCR mutagenesis
ligate with pBluescript SK

pAP 227 linker
(MAL-C variant)

CAGGTGTTACAGGGGAAGCGATATCAGTTACTATG
GTCCACCAATGTCCCCCTTCGCTATAGTCAATGATAC

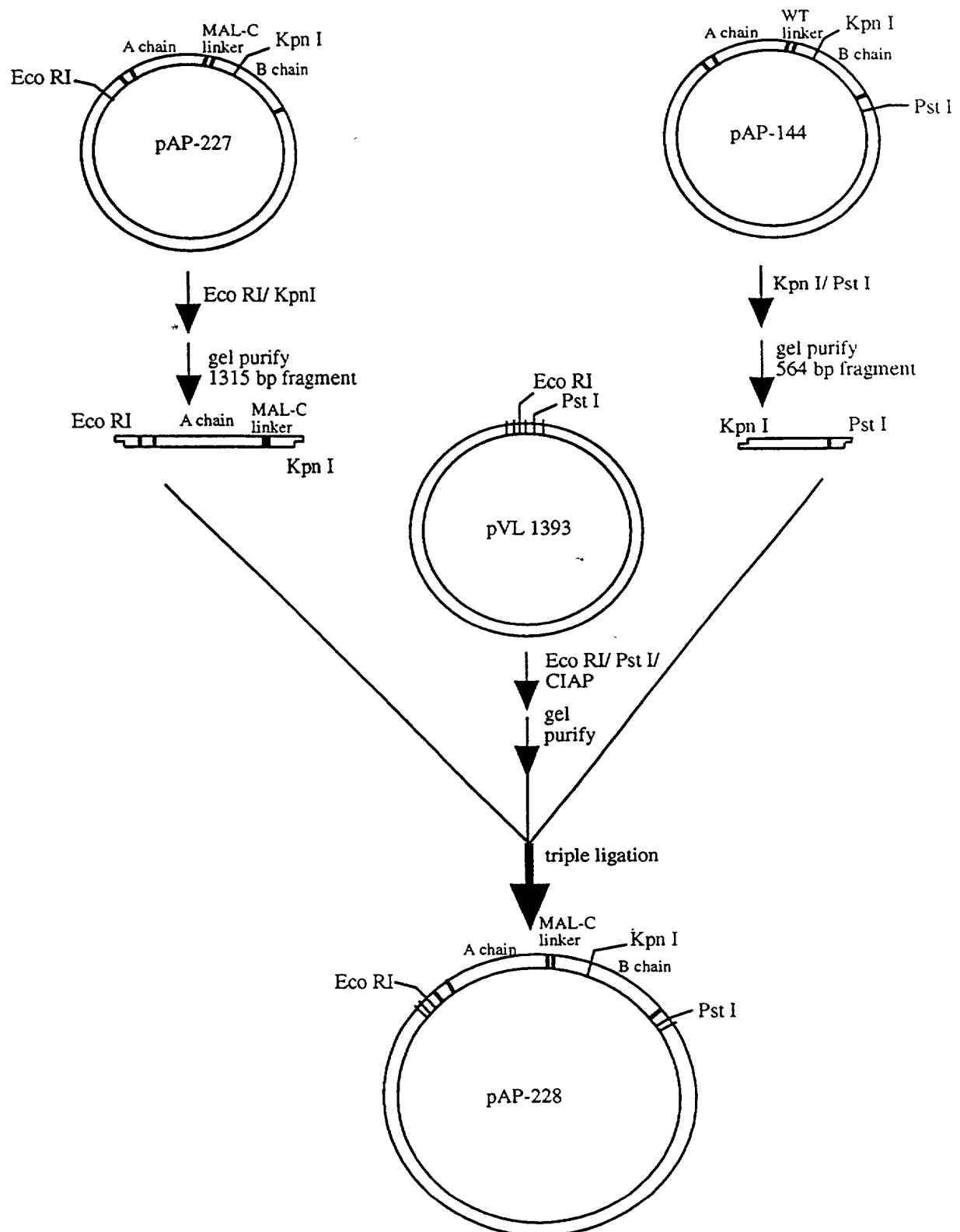
FIGURE 9C

FIGURE 9D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAAATGCGCACGAGAAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCAGGTGGTTACAGGGGAAGCGATATCAGTTACTATGGC
AGCAGTGTCAAAGTCCACCAATGTCCCTTCGCTATAGTCAATGATACCG

FIGURE 9D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAACAGT
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCTGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

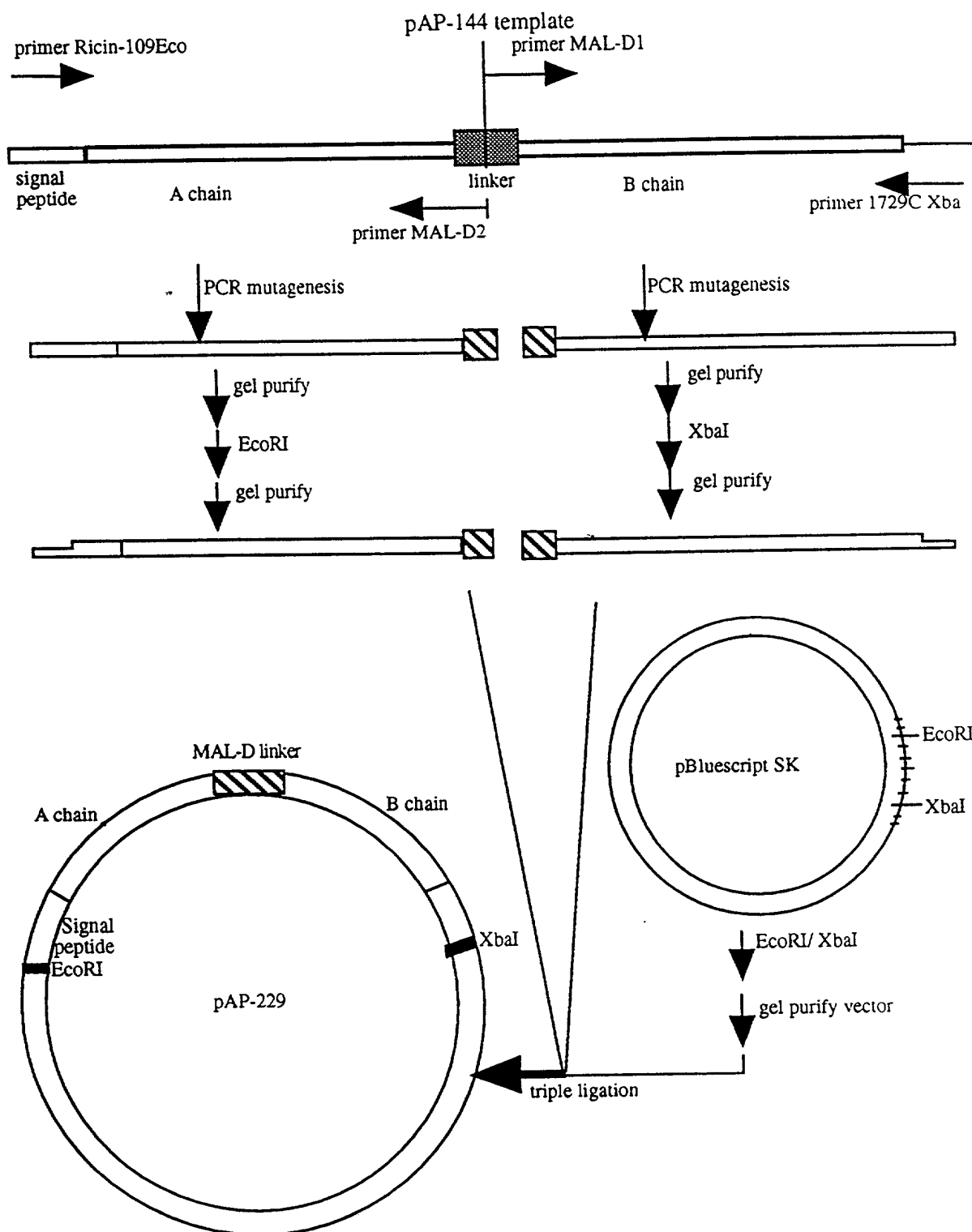
FIGURE 10A

FIGURE 10B

WT preprorin linker

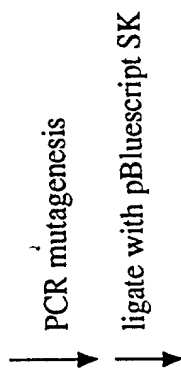
primer MAL-D1

5' - CTGTCGTTCCCTACTAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCCAATTTTAAT
AGAAACGAATATTCGGTCAACCACGGTTTAAATAA

3' - GGTAGCAGTGTCAAACGAAACCTCTCTTGCAAG -5'

primer MAL-D2

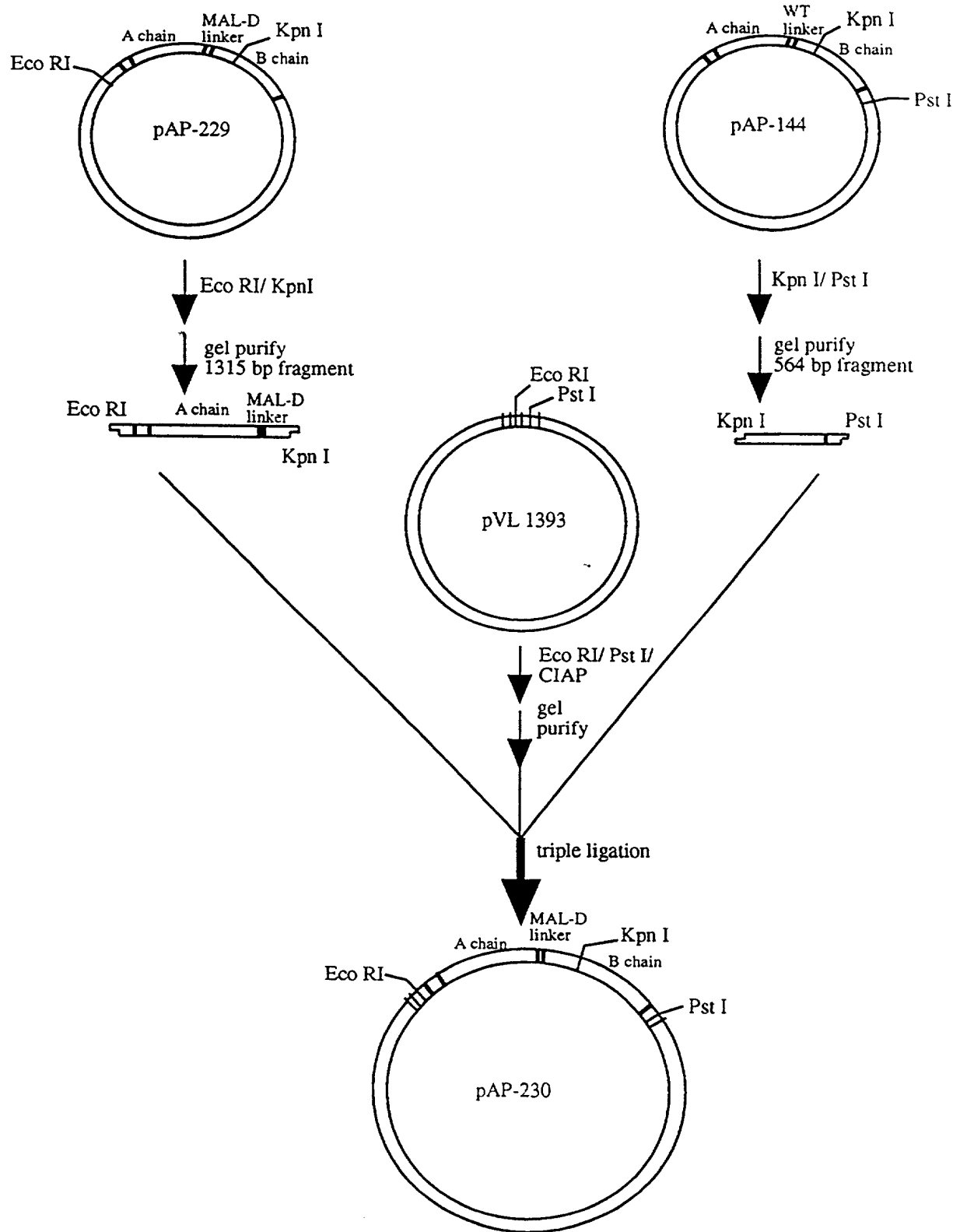


pAP 229 linker
(MAL-D variant)

GCTTTGGAGAGAACGTTCCCTGTCGTTCCCTACTAAT
CGAAACCTCTCTTGCAAGGACAGCAAGGATGATTA

49/254

FIGURE 10C



0954154 044400

FIGURE 10D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTGCG
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTAAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTGA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGCTTTGGAGAGAACGTTCTGTGCTTCCCTACTAATGC
AGCAGTGTCAAACGAAACCTCTCTTGCAAGGACAGCAAGGGATGATTACG

FIGURE 10D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACCAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTTGTACCCGAGAAAATACGTCACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGACAGTGTGTGTGCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

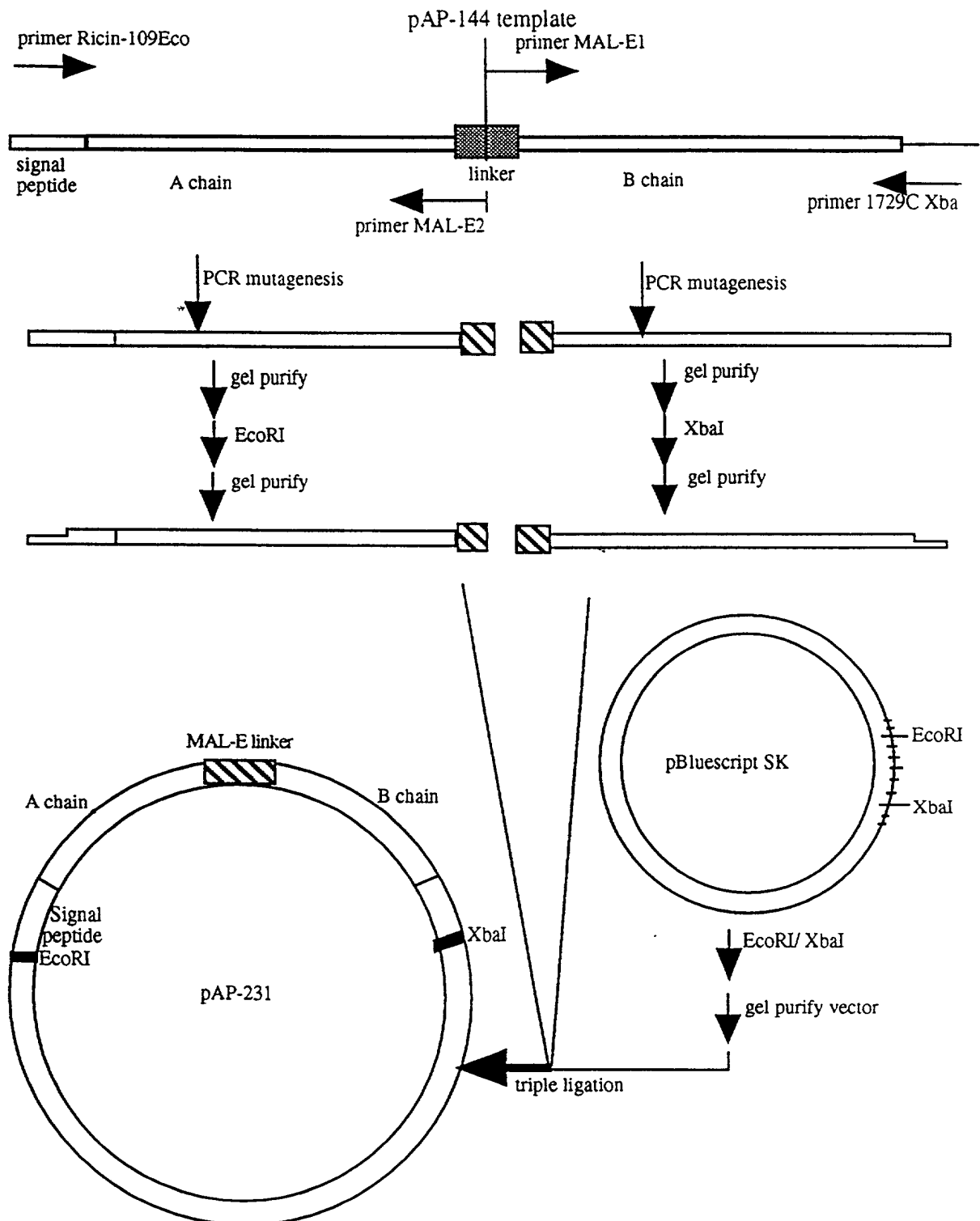
FIGURE 11A

FIGURE 11B

WT preporicin linker

primer MAL-E1

5' - AATAATTCACAGCATCAGGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGGAATATCCGGTCACCCAGGTTTAAATTA

3' - GGTAGCAGTGTCAAATTTAAGGTTCTATACGAT -5'

primer MAL-E2

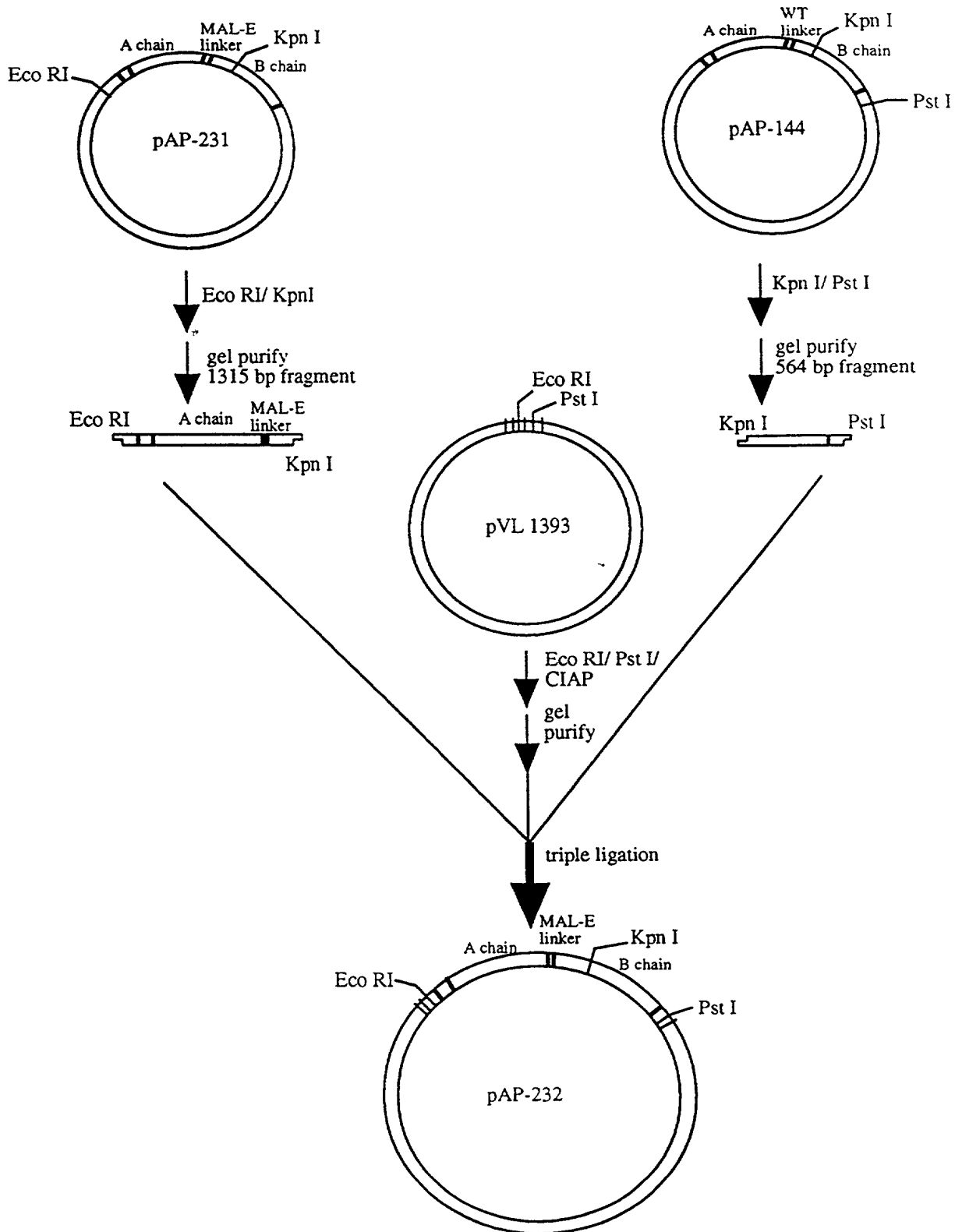
PCR mutagenesis
ligate with pBluescript SK

pAP 231 linker
(MAL-E variant)

AAATTCAGATATGCTAAATAATTCACAGCATCAG
TTTAAGGTTCTATACGATTATTAAGTGTGCTAGTC

54/254

FIGURE 11C



004440"TS1560

FIGURE 11D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCTCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAAGTTT

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTTGGT

901 TCGTCACAGTTTAAATTCCAAGATATGCTAAATAATTCACAGCATCAGGC
AGCAGTGTCAAATTTAAGGTTCTATACGATTTATTAAGTGTGCTAGTCCG

FIGURE 11D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAACGCAATA
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCTCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

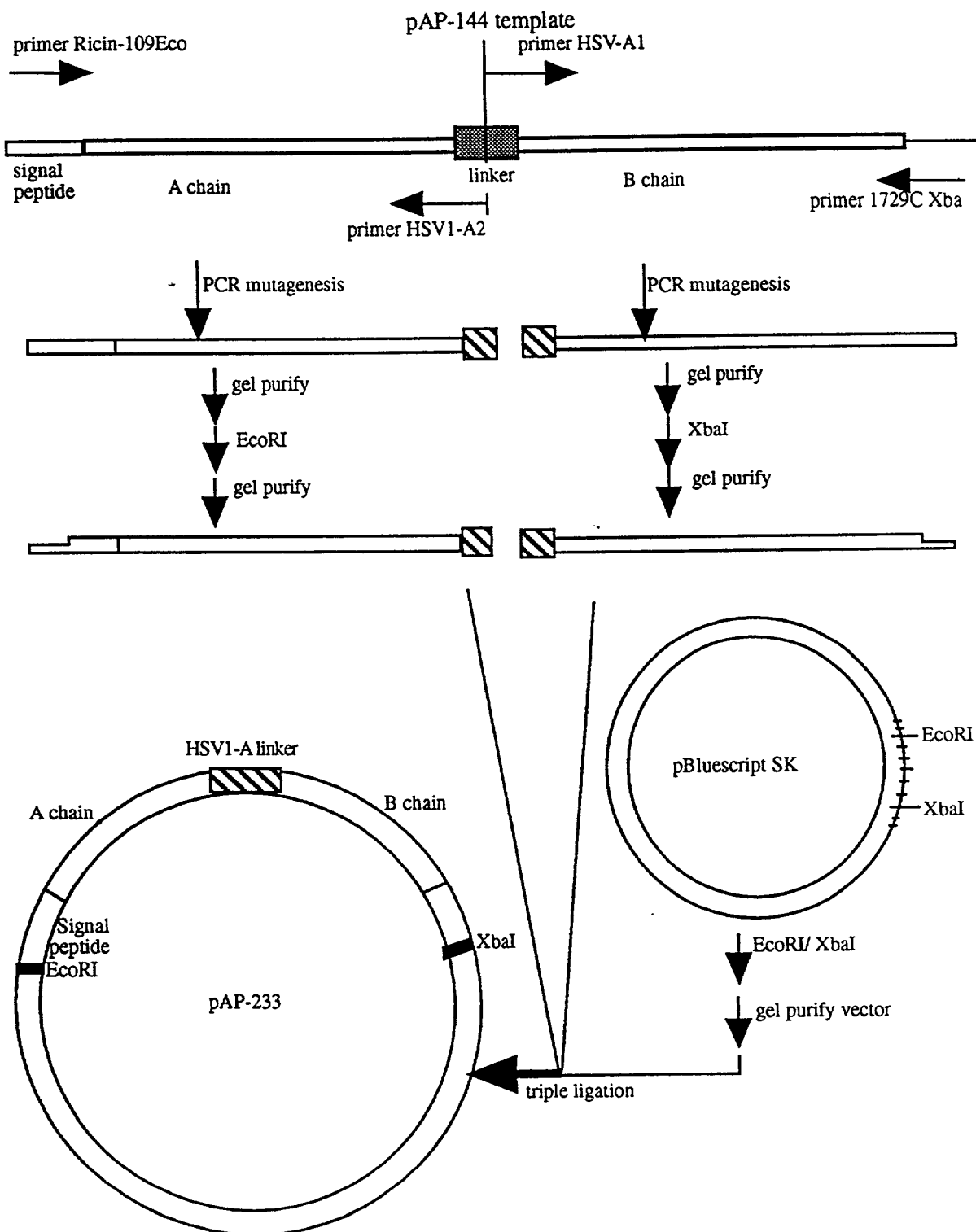
FIGURE 12A

FIGURE 12B

WT preprorin linker

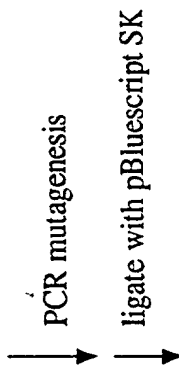
primer HSV1-A

5' - TCGTCGGGCACATGTTAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCGGT CACCACGGTTAAAAATTA

3' - AGCAGTGTCAAAAAGACGCGAACATTTGCGT-5'

primer HSV1-A



pAP 233 linker
(HSV1-A variant)

TCTGCGCTTGTAACGCATCGTCGGCACATGTTAAT
AGACGGAACATTTGCGTAGCAGCCGTTACAATTA

FIGURE 12C

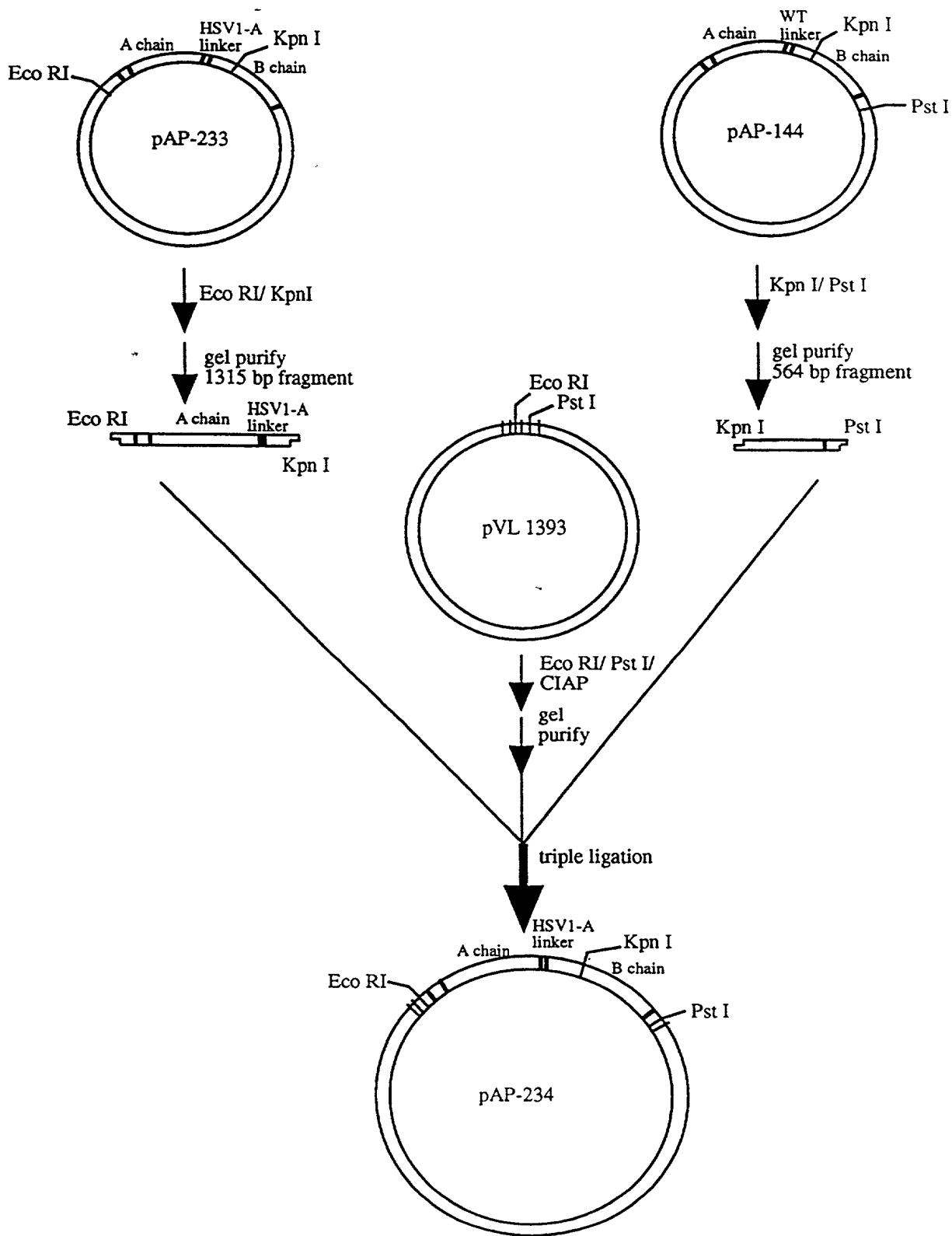


FIGURE 12D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTTCGCGG
CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA
AGCAAATGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTTCGGCTACCGTGTGGAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTT

451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCCTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAACGTTAGGTTTACTAAAGTCTTCGTCTGTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTGCGCTTGTAACGCATCGTCGGCACATGTTAATGC
AGCAGTGTCAAAGACGCGAACATTTGCGTAGCAGCCGTGTACAATTACG

FIGURE 12D (CONT'D)

951 TGATGTTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTGCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTTGCACCCGAGAAATACGTCACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTGTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGTATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAAGTGTGTGTCTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

62/254

FIGURE 13A

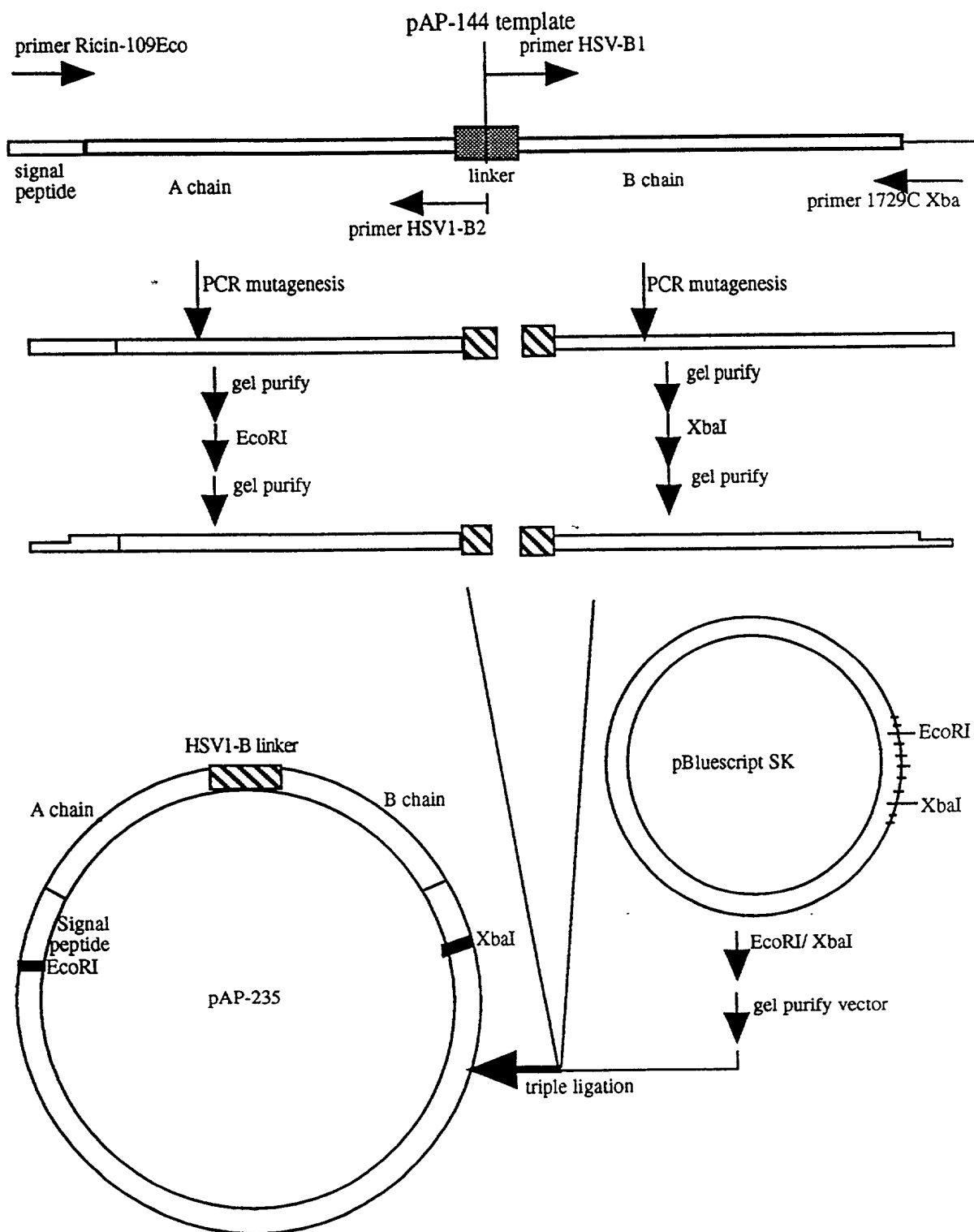


FIGURE 13B

WT preprorin linker

primer HSV1-B

5' - TCGGAGAAATTTAAGAAATGCTGATGTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCGGTCACCCACGGTTTAAATTA

3' - AGCAGTGTCAAAAGATGCATAAATGTCCGT -5'

primer HSV1-B

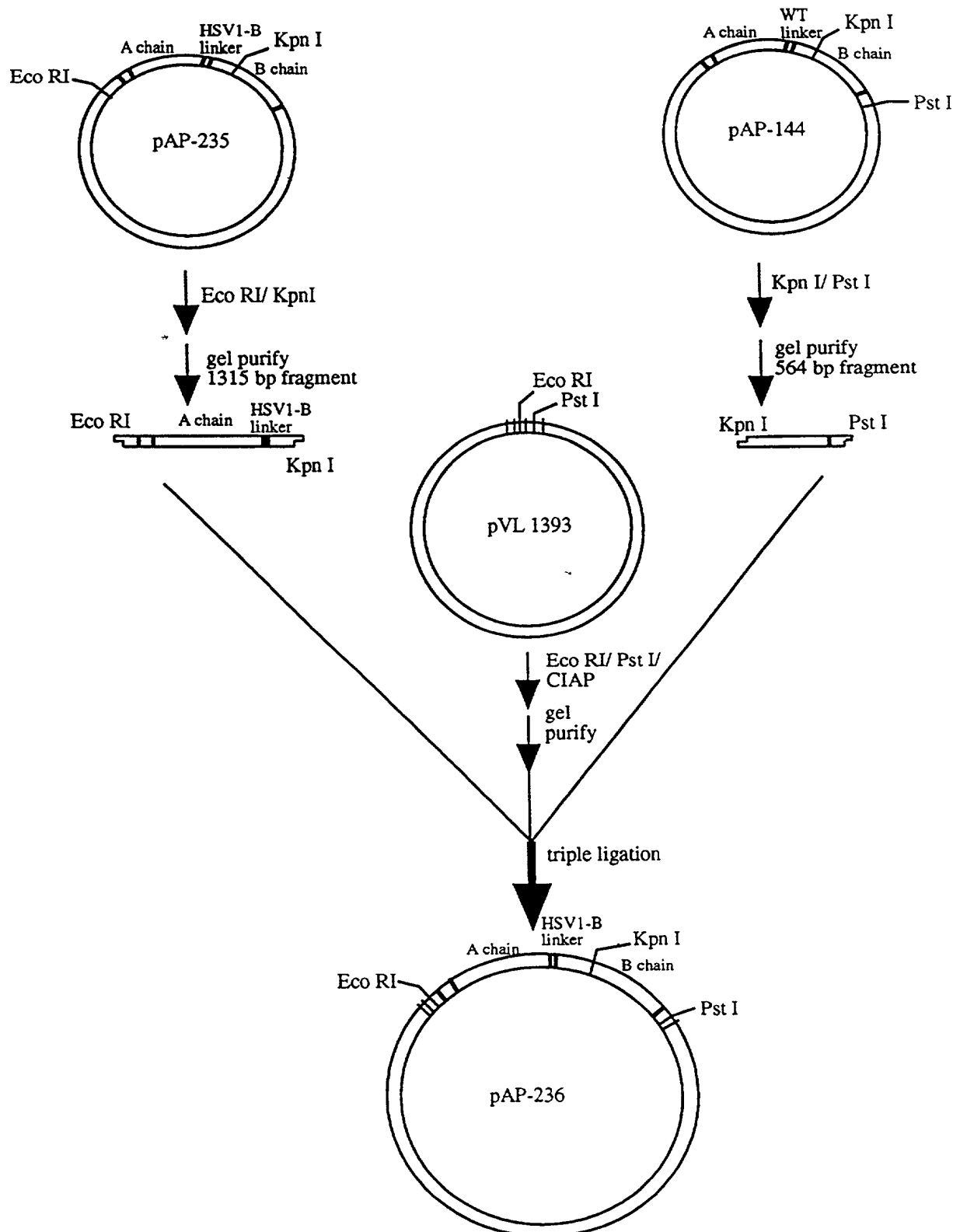
PCR mutagenesis
ligate with pBluescript SK

pAP 235 linker
(HSV1-B variant)

TCTACGTATTACAGGCATCGGAGAAATTTAAGAAAT
AGATGCATAAATGTCCGTAGCCTCTTAAATCTTA

64/254

FIGURE 13C



004440" 6544560

65/254

FIGURE 13D

10 20 30 40 50
1 GAATTCATGAAACCGGGAGGAAATAC'TATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGBAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
101 AGGATAACAACATATTCGCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTGA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAGAAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAAGTCTTATCAACCCCTCT
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
901 TCGTCACAGTTTTCTACGTATTTACAGGCATCGGAGAAATTTAAGAATGC
AGCAGTGTCAAAGATGCATAAATGTCCGTAGCCTCTTTAAATCTTACG

FIGURE 13D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGA CTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCTCAG
 TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACAGGACGTA CTTTTATCTACCGAATTTATTTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCTGTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

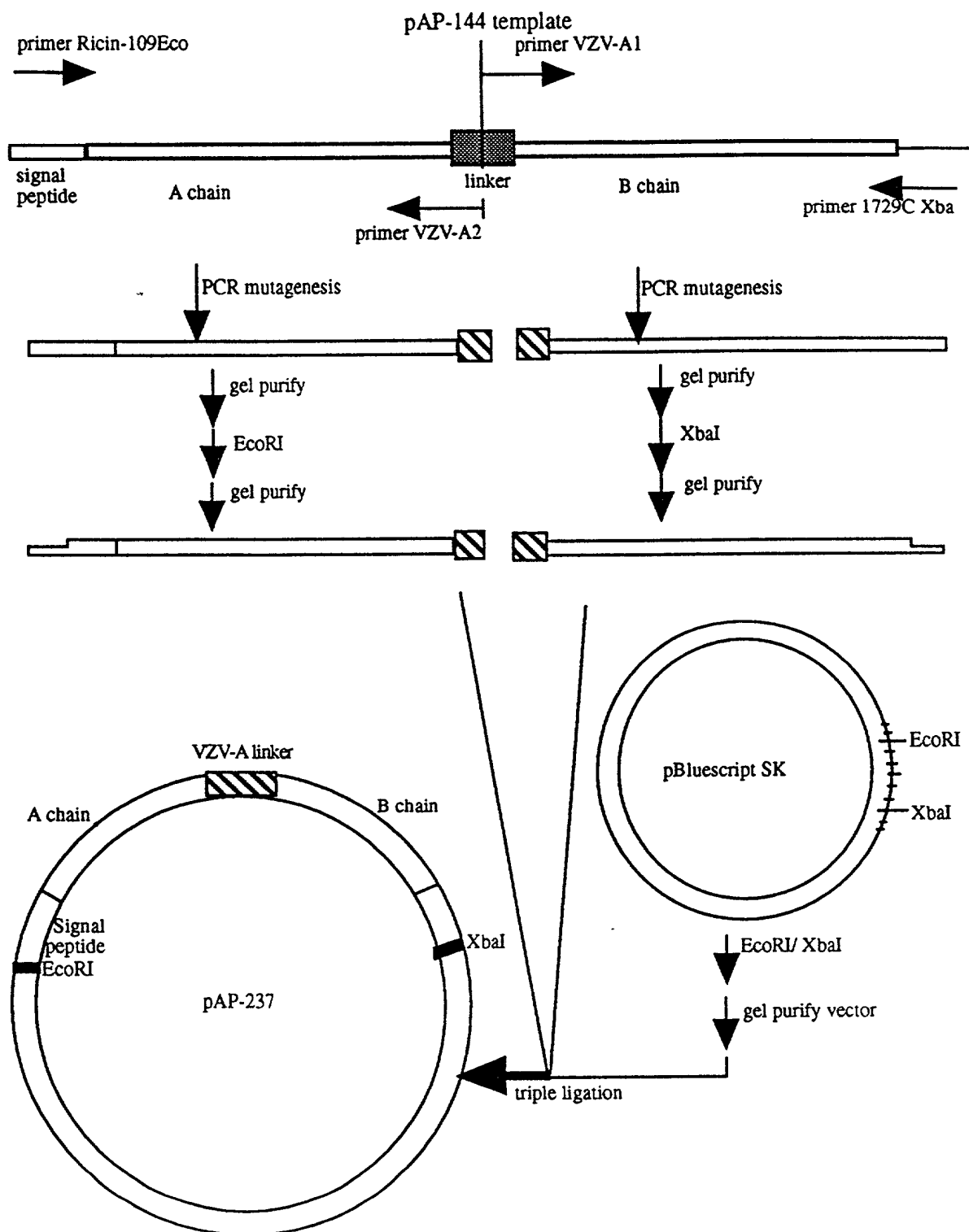
FIGURE 14A

FIGURE 14B

WT preprorin linker

primer VZV-A1

5' - GTGGAGGCAAGTTCTAATGCTGATGTTTGT -3'

TCCTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCTGGTCAACACGGTTTAAATTA

3' - AGCAGTGTCAAAAAGAGTCCTACATTTGCGT-5'

primer VZV-A2

PCR mutagenesis
ligate with pBluescript SK

pAP 237 linker
(VZV-A variant)

TCTCAGGATGTAAACGCAGTGGAGGCAAGTTCTAAT
AGATCCTACATTTGCGTCACTCCGTTCAAGATTA

69/254

FIGURE 14C

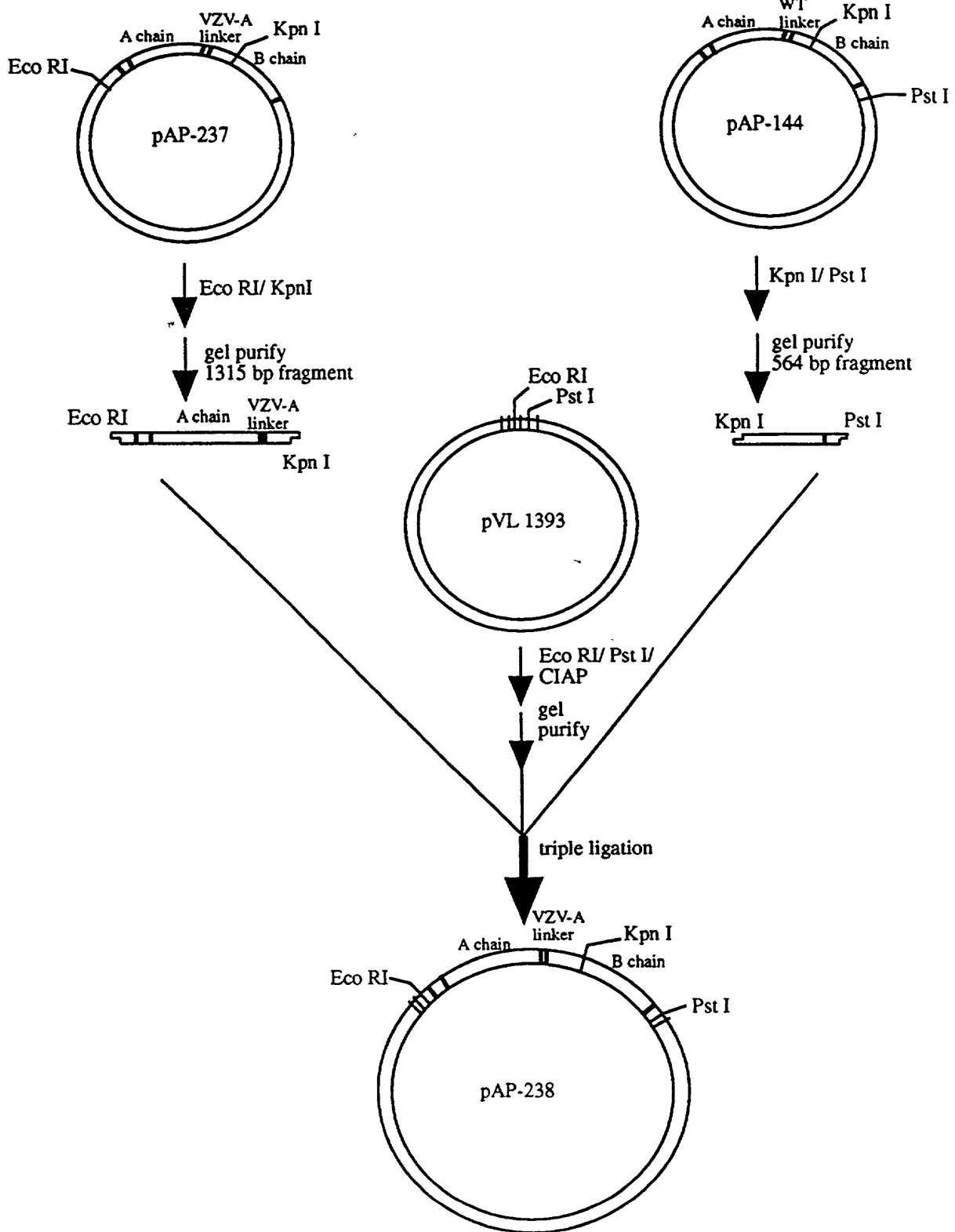


FIGURE 14D

10 20 30 40 50
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
 CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACCTCTCA
 TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTTCTGTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
 ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
 TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTTA
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGCG
 GCTATATGTAAGCGGAAACCAACCATTAATACTATCTGAAGTGTGTTGAACG
 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACCT
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
 AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTTGGT
 901 TCGTCACAGTTTTCTCAGGATGTAAACGCAGTGGAGGCAAGTTCTAATGC
 AGCAGTGTCAAAGAGTCCTACATTTGCGTCACCTCCGTTCAAGATTACG

FIGURE 14D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGTATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTCTGCCATGAAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

72/254

FIGURE 15A

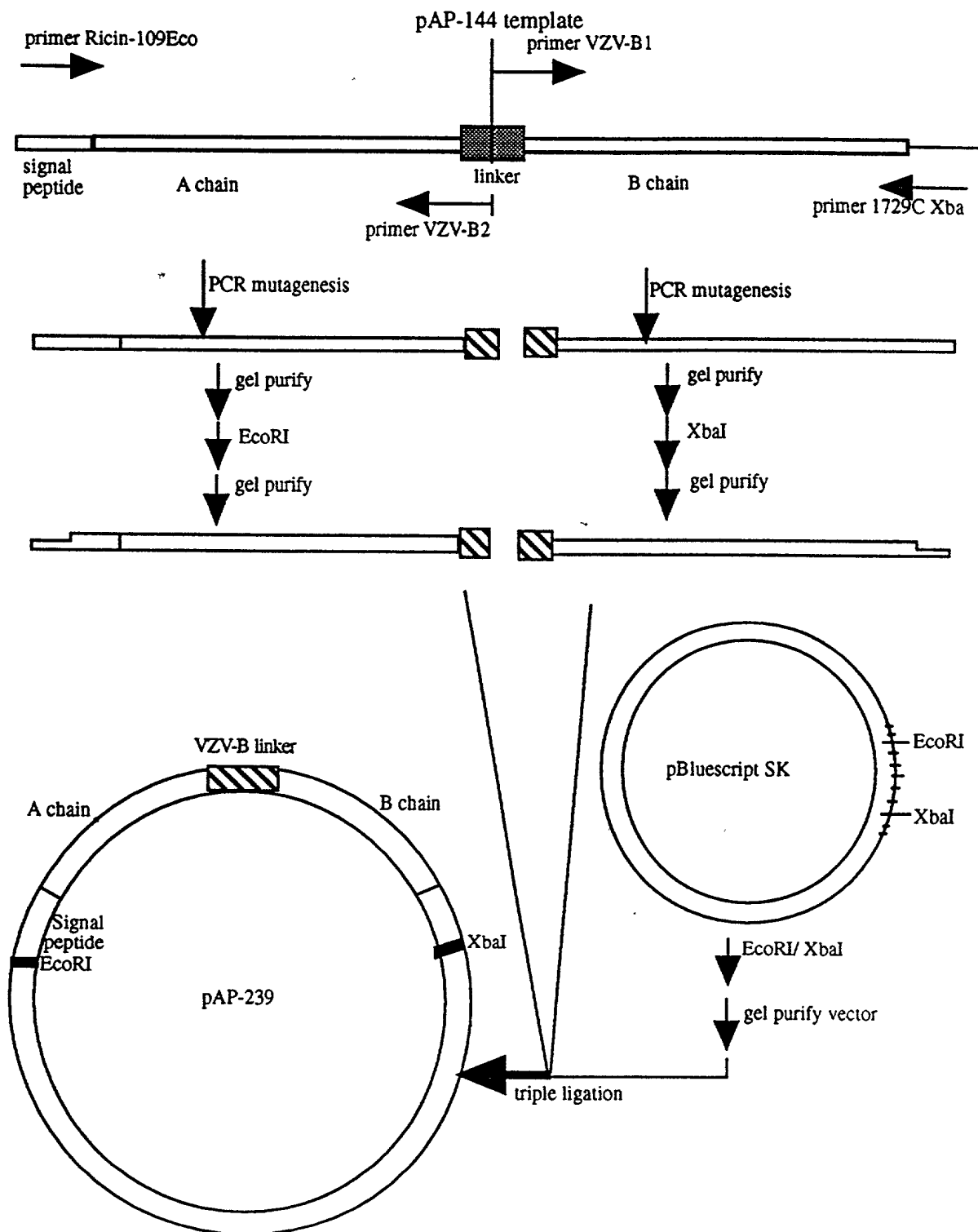


FIGURE 15B

WT preprorin linker

primer VZV-B1

5' - TCGACGGGATATGGTAATGCTGATGTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCGGTCACCCAGGTTAAAAATTA

3' - AGCAGTGTCAAAAGACACATAAAATGTCCGT-5'

primer VZV-A2

PCR mutagenesis

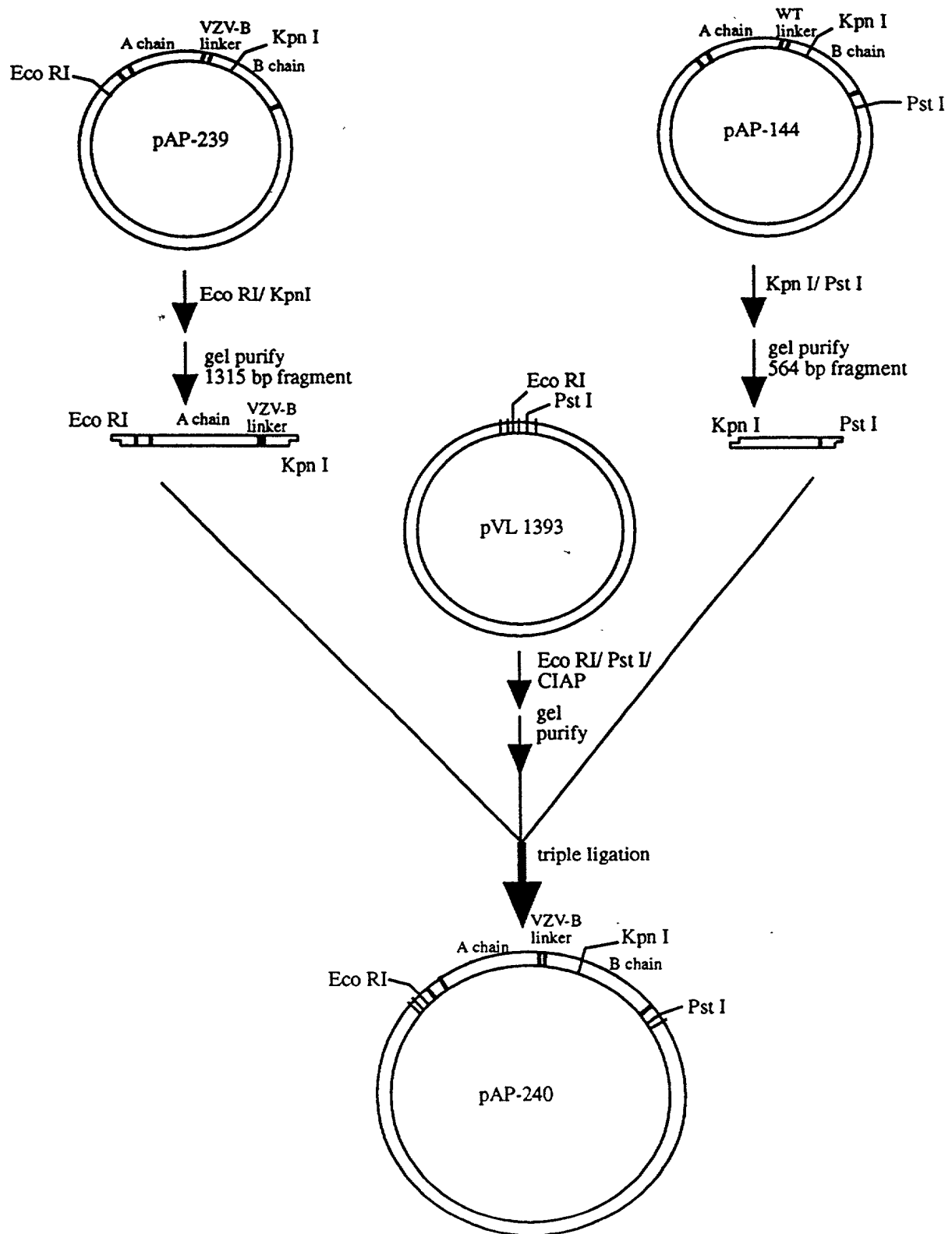
ligate with pBluescript SK

pAP 239 linker
(VZV-B variant)

TCTGTGTATTACAGGCATCGACGGGATATGGTAAT
AGACACATAAATGTCCGTAGCTGCCCTATACCATTA

74/254

FIGURE 15C



055151 04400

FIGURE 15D

10 20 30 40 50
 1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
 CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
 CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC
 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
 TCCTATTGTTGTATAAGGGGTTTGTATTGGGTAAATATTTGAAATGGTGT
 151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
 CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
 201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA
 AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
 251 ACAGAGTTGGTTTGCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA
 TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
 301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
 TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
 ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT
 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
 TAGTCCCTTCTACGTCTTCGTTAGTGAGTAGAAAAAGTGACTACAAGTTTAA
 451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
 GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
 501 TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
 ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
 GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
 601 CTGGCTCGTTCCCTTTATAATTGTCATCCAAATGATTTTCAAGCAGCAAG
 GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA
 TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAAATAGTTGGGGGAGA
 CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
 GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA
 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
 AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
 ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
 901 TCGTCACAGTTTTCTGTGTATTTACAGGCATCGACGGGATATGGTAATGC
 AGCAGTGTCAAAGACACATAAATGTCCGTAGCTGCCCTATACCATTACG

FIGURE 15D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTTGTACACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

FIGURE 16A

PCR Mutagenesis of Preproricin Gene to Create an EBV-A Variant
Gene a) Cloning Strategy

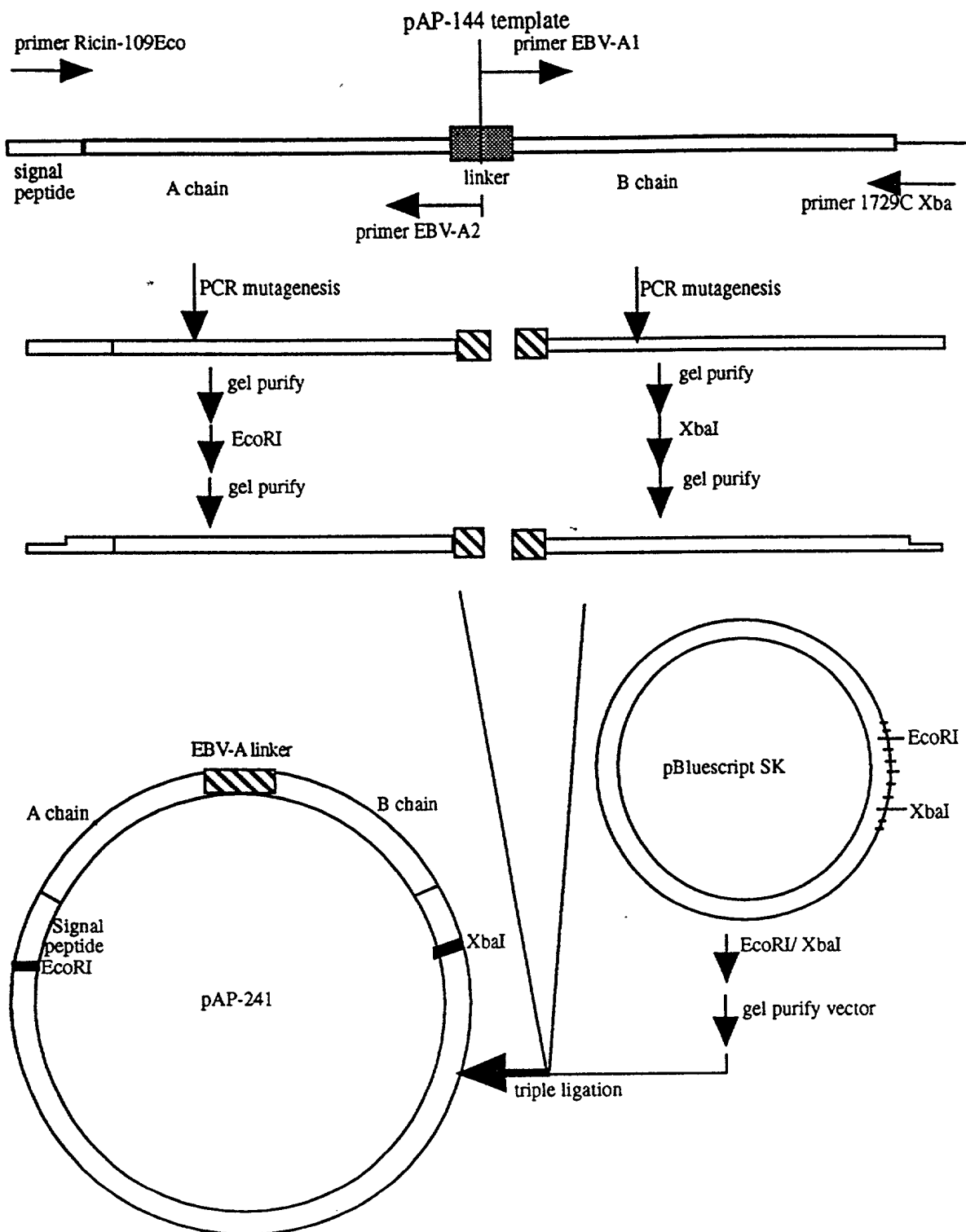


FIGURE 16B

WT preprorcin linker

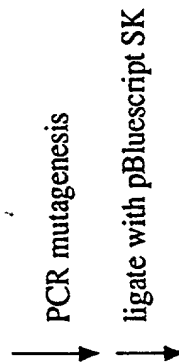
primer EBV-A1

5' - TCGGCGTCAGGTGTTAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCCAATTTTAAT
AGAAACGAATATTCGGTCACCCAGGTTAAATTA

3' - AGCAGTGTCAAAAGATTTCGAACATGTCCGT -5'

primer EBV-A2

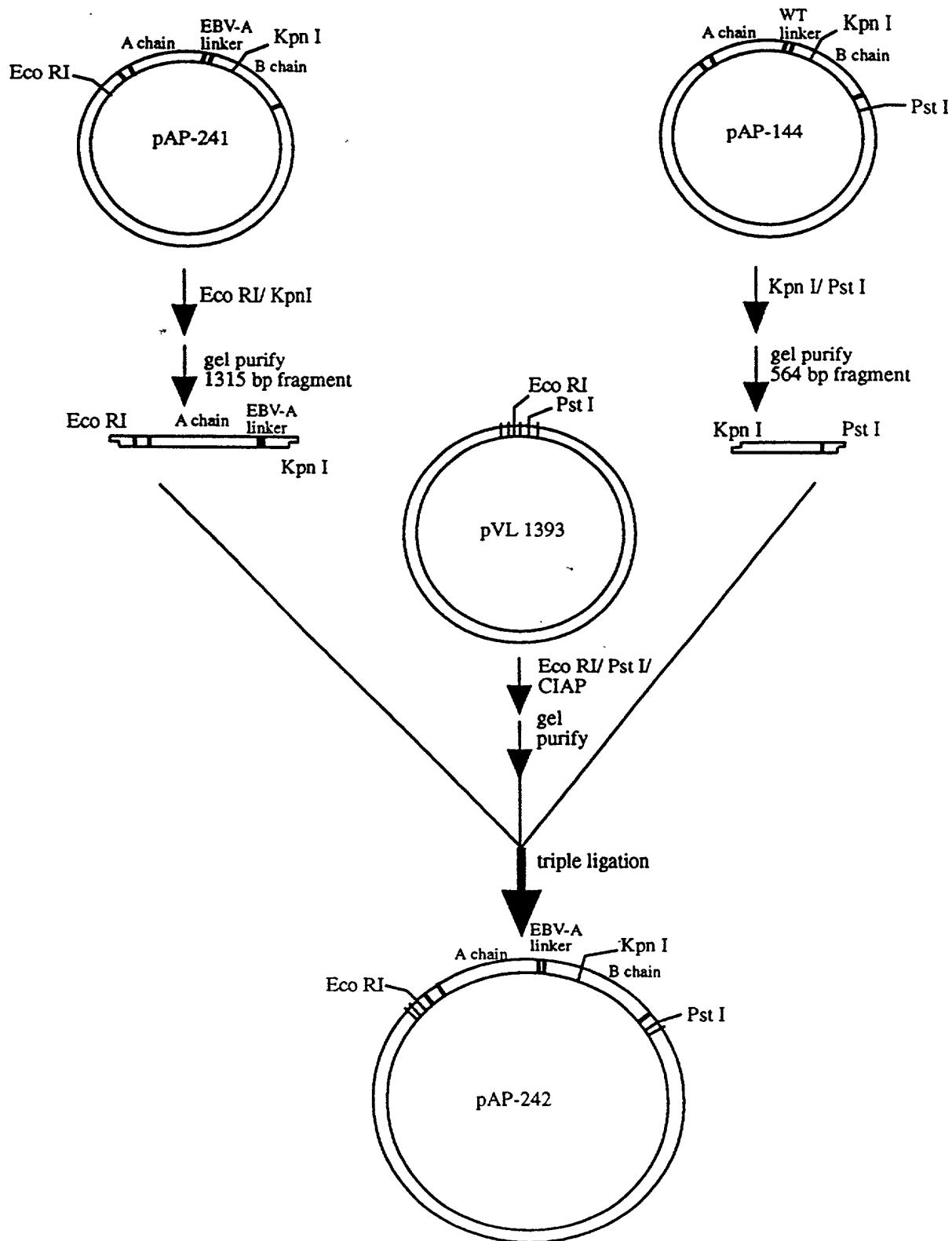


pAP 241 linker
(EBV-A variant)

TCTAAGCTTGACAGGCATCGGCGTCAGGTGTTAAT
AGATTGGAACATGTCCGTAGCCGAGTCCACAATTA

79/254

FIGURE 16C



204440 " 151560

FIGURE 16D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTGTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTTTA

451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCAATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCTTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT

901 TCGTCACAGTTTTCTAAGCTTGTAAGGCATCGGCGTCAGGTGTTAATGC
AGCAGTGTCAAAAGATTGGAACATGTCCGTAGCCGCAGTCCACAATTACG

FIGURE 16D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAACGCAATA
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGAATACGTTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTATGACGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAACCAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTGCTGCGCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

82/254

FIGURE 17A

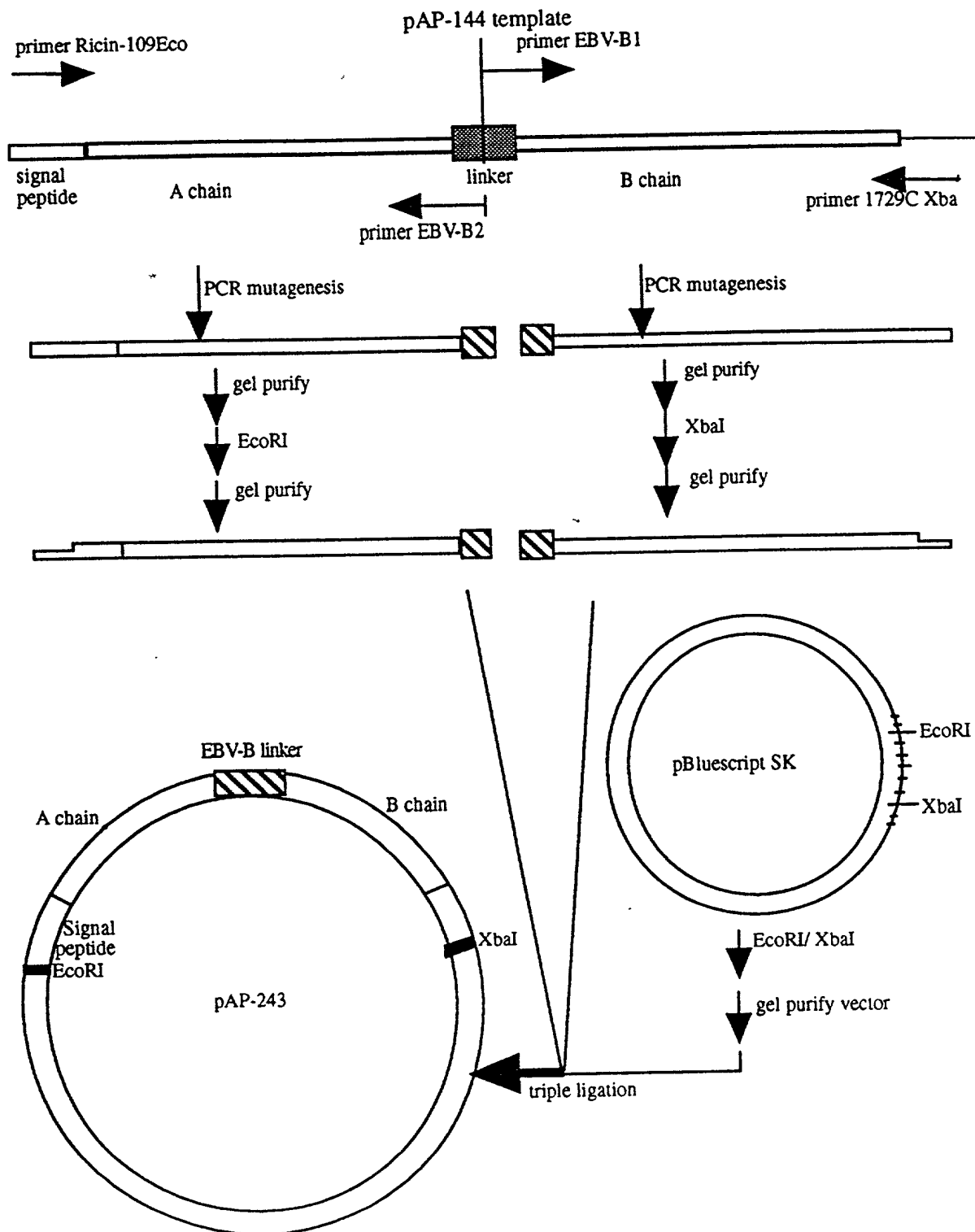


FIGURE 17B

WT preprorin linker

primer EBV-B1

5' - TCGGACGCACCTGATAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCGGTCACCCACGGTTAAAATTA

3' - AGCAGTGTCAAAAGAACATAGATTTCGGT-5'

primer EBV-B2

PCR mutagenesis

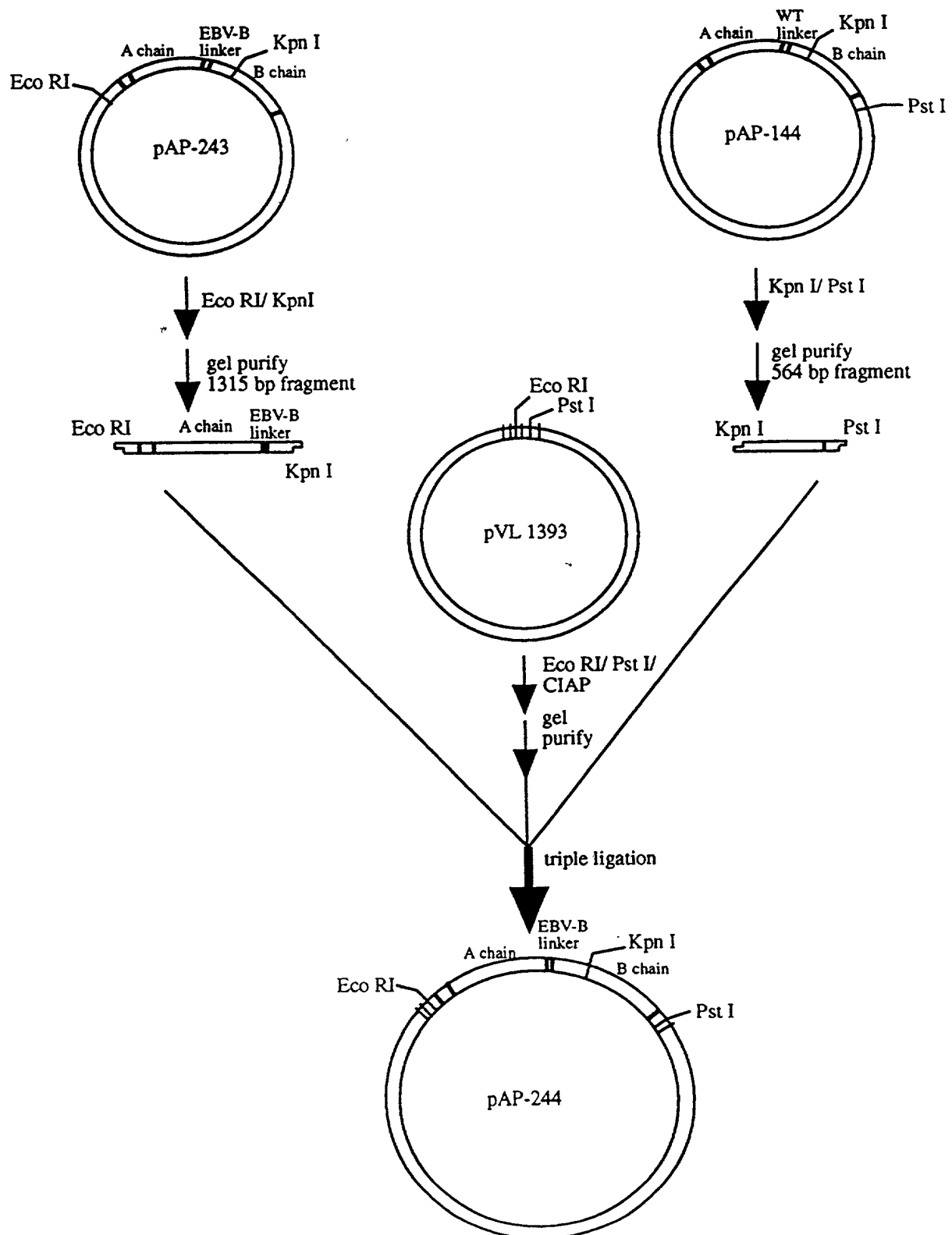
ligate with pBluescript SK

pAP 243 linker
(EBV-B variant)

TCTTCGTATCTAAAGGCATCGGACGCACCTGATAAT
AGAACATAGATTTCGGTAGCCTGCCGTGGACTATTA

84/254

FIGURE 17C



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85/254

FIGURE 17D

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTTCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACAACGGTTTATTTTAGTTGAACCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA				
	ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAACAAGTTTGA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC				
	GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA				
	CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTTATCAACCCCTCT				
751	CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT				
	GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA				
801	TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA				
	AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT				
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA				
	ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT				
901	TCGTACAGTTTCTTCGTATCTAAAGGCATCGGACGCACCTGATAATGC				
	AGCAGTGTCAAAGAAGCATAGATTTCGCTAGCCTGCGTGGAATATTACG				

FIGURE 17D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCCTGCAAGTGTGTGTGTCTGCGCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

87/254

FIGURE 18A

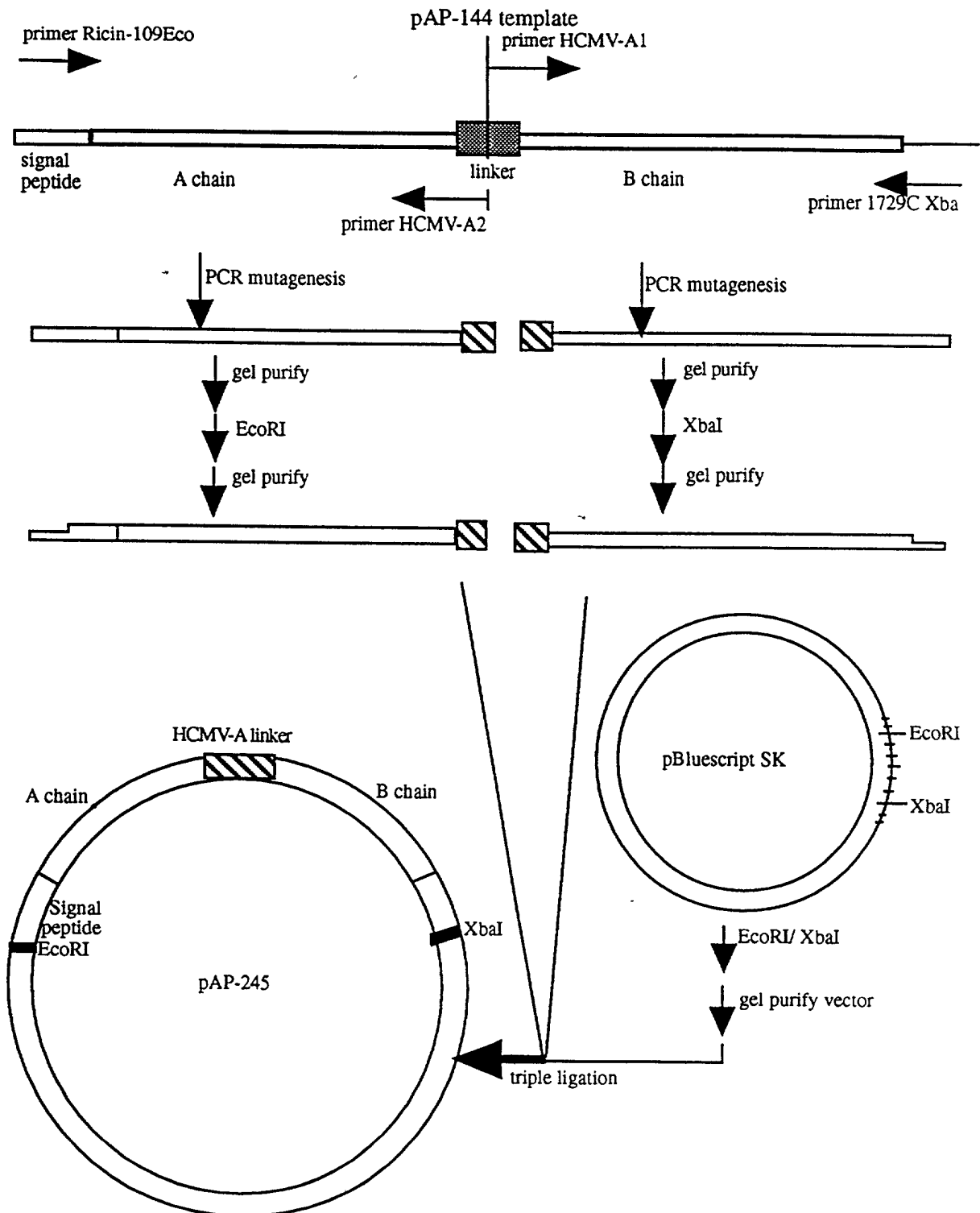


FIGURE 18B

WT preprorin linker

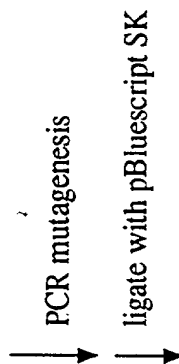
primer HCMV-A1

5' - TCGGTAGACTTGCTAATGCTGATGTTGT -3'

TCTTTGCTTATAAGGCCACTGGTGCCAAATTTTAAT
AGAAACGAATATTCCGGTCACCCACGGTTAAATTA

3' - AGCAGTGTCAAAAGACCCCAACATTTACGT-5'

primer HCMV-A2

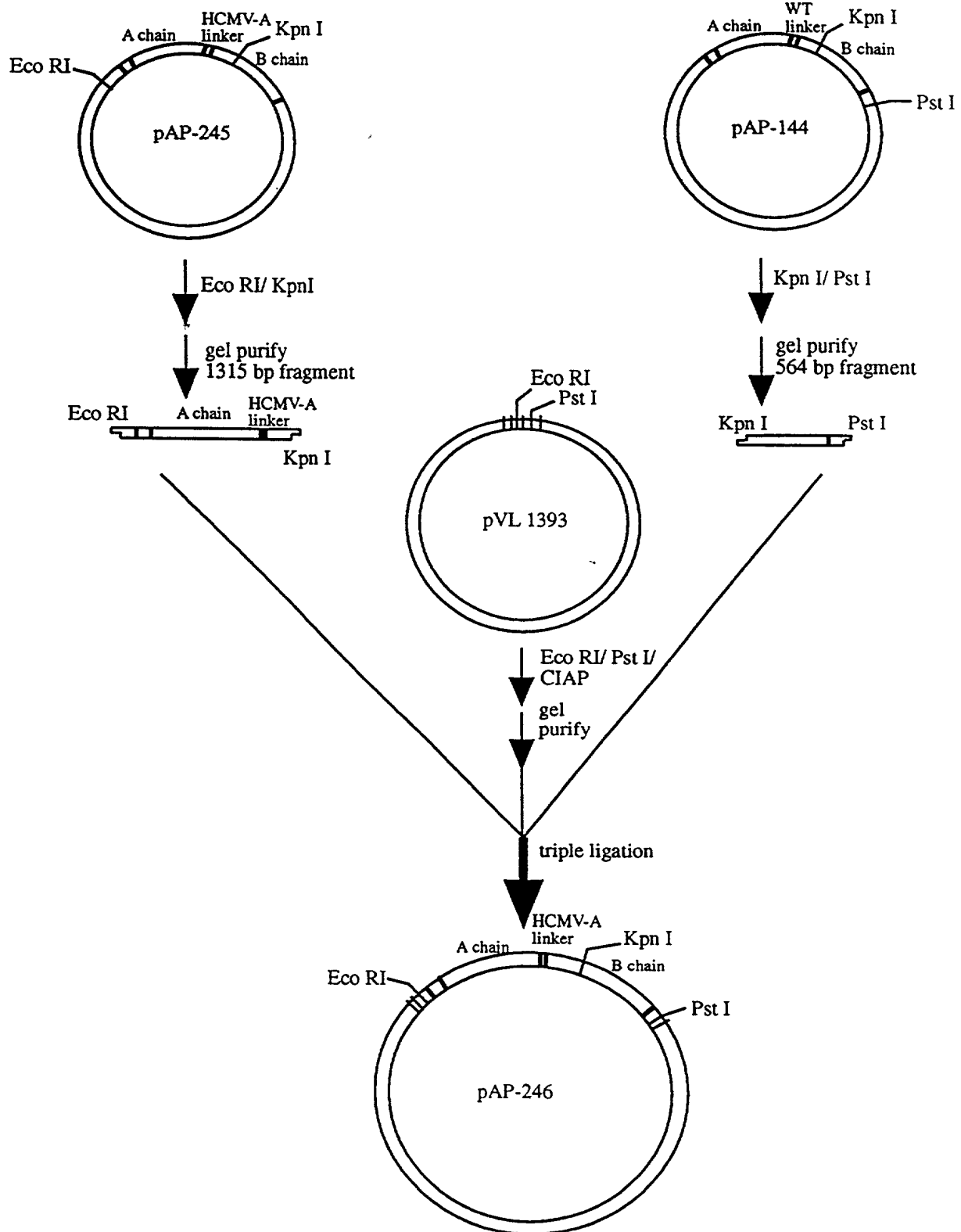


pAP 245 linker
(HCMV-A variant)

TCTGGGTTGTAAATGCATCGGTGAGACTTGCTAAT
AGACCCCAACATTTACGTAGCACATCTGAACGATTA

89/254

FIGURE 18C



004740" 1511550

90/254

FIGURE 18D

10 20 30 40 50
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA
AGCAAATGTTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGCAGCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
501 TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAT
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
901 TCGTCACAGTTTTCTGGGGTTGTAAATGCATCGTGTAGACTTGCTAATGC
AGCAGTGTCAAAGACCCCAACATTTACGTAGCACATCTGAACGATTACG

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FIGURE 18D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGC GTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAACCATTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

92/254

FIGURE 19A

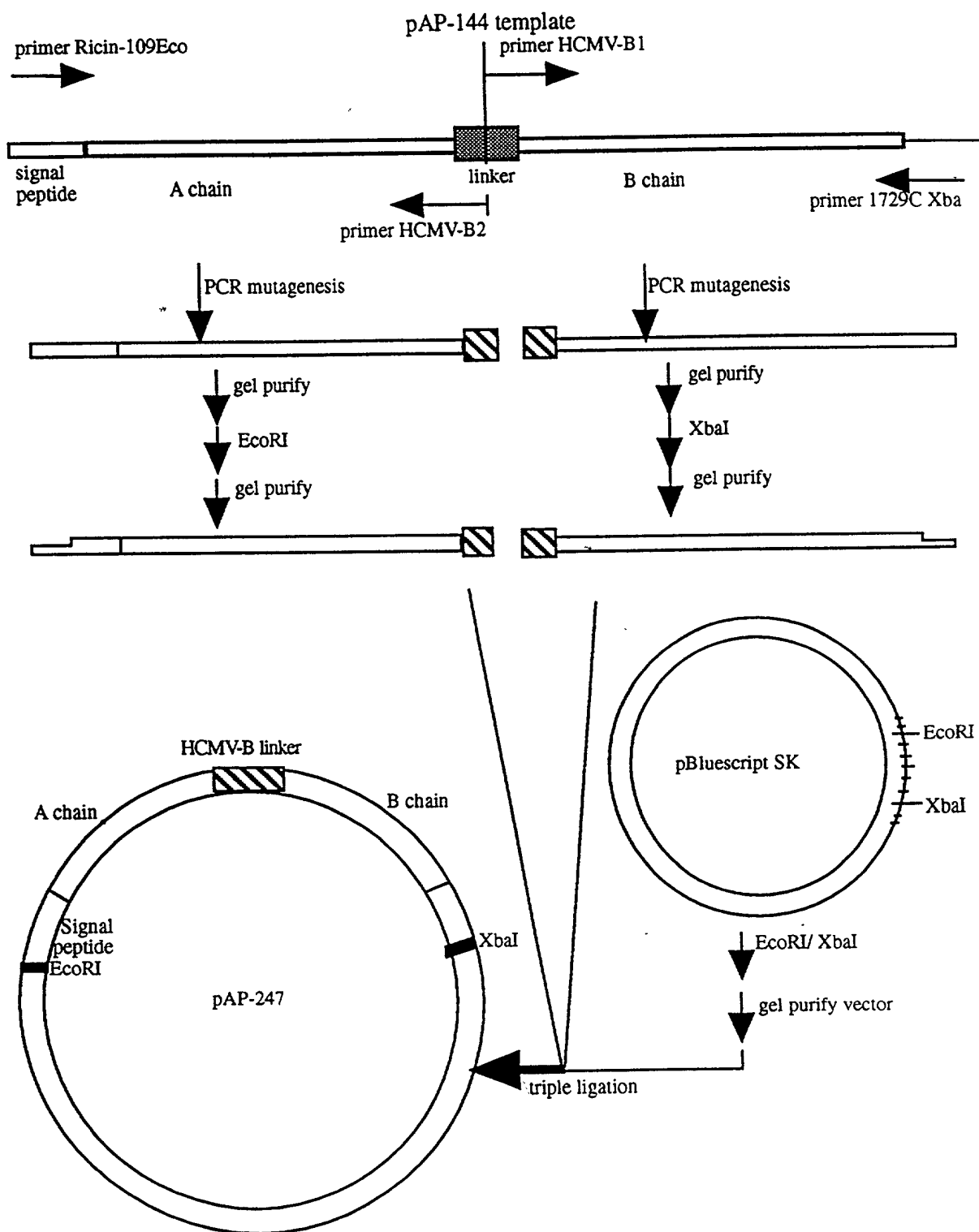


FIGURE 19B

WT preprorcin linker

primer HCMV-B1

5' - TCGGTGTCACCTGAAAAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCTGGTACCCACGGTTTAAATTA

3' - AGCAGTGTCAAAAGAGCATACATTTCCGT-5'

primer HCMV-B2

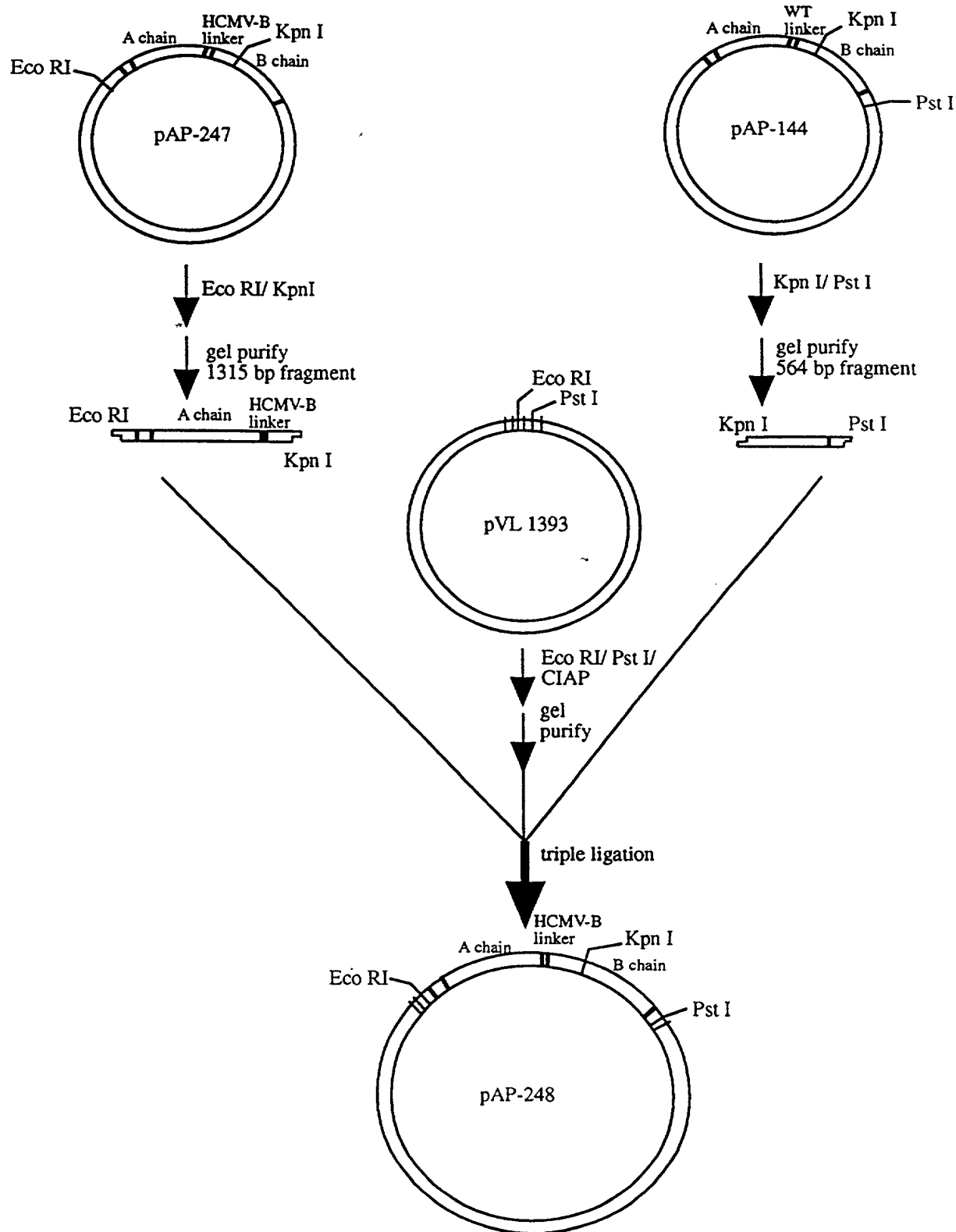
PCR mutagenesis
ligate with pBluescript SK

pAP 247 linker
(HCMV-B variant)

TCTTCGTATGTAAAGGCATCGGTGTCACCTGAAAT
AGAGCATACATTTCCGTAGCCACAGTGGACTTTA

94/254

FIGURE 19C



95/254

FIGURE 19D

10 20 30 40 50
1 GAATTCATGAAACCGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAAGTGTAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCGCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCCTATAAACCAACGGTTTATTTAGTTGAACCTCTCA
TGTCTCAACCAACGGATATTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAACAAAGTTTCA
451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTATCAACCCCTCT
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCTCGGAAACGATCAGGTTA
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
901 TCGTCACAGTTTTCTTCGTATGTAAAGGCATCGGTGTCACCTGAAAATGC
AGCAGTGTCAAAGAAGCATAACATTTCCGTAGCCACAGTGGACTTTTACG

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FIGURE 19D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTGTGCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTGTCACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAAACGGAATGTTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGTATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

97/254

FIGURE 20A

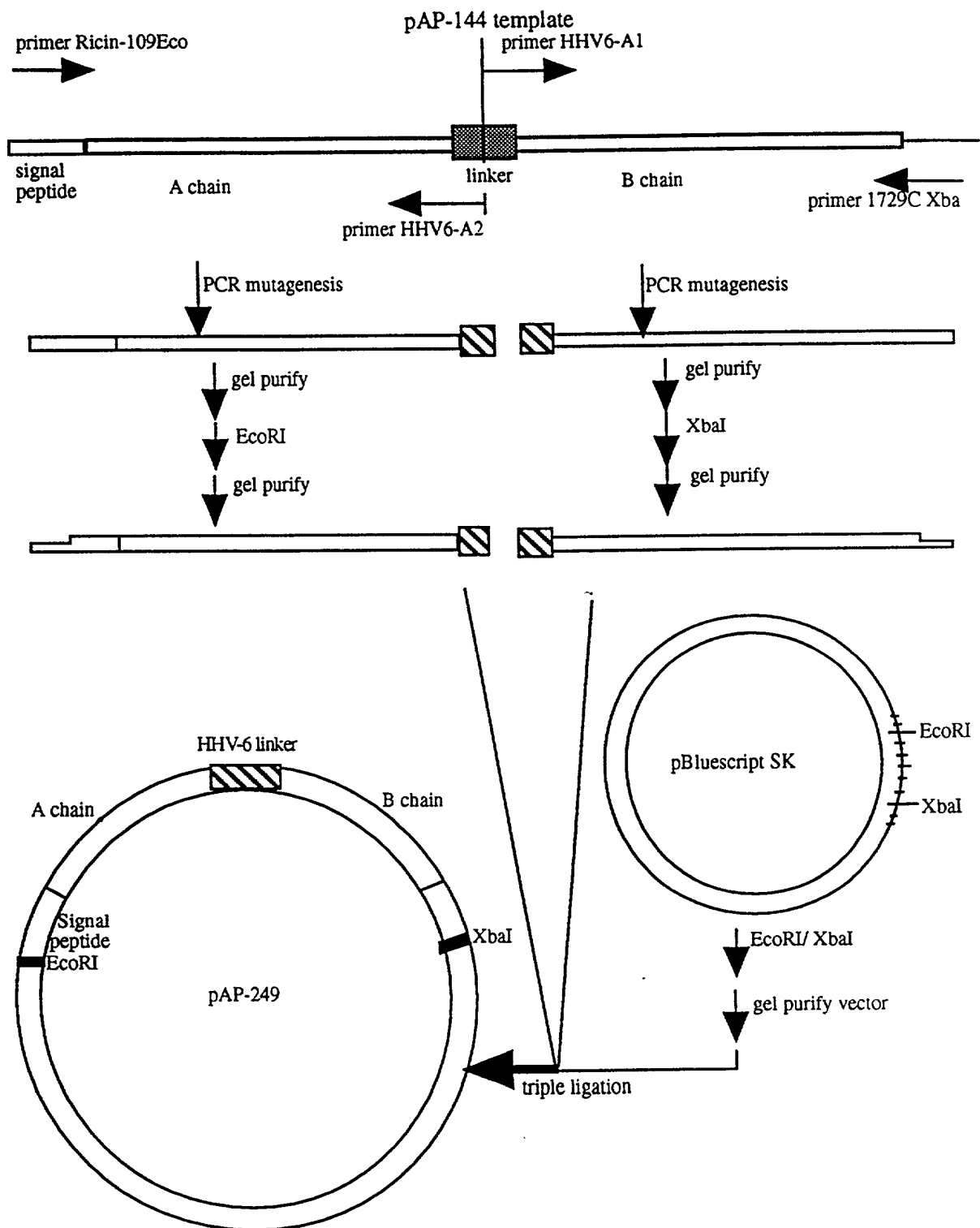


FIGURE 20B

WT preproricin linker

primer HHV6-A1

5' - TCGGTGCCAAATTTTAAT -3'

TCCTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAAATATCCGGTCACCCACGGTTAAAAATTA

3' - AGCAGTGTCAAAAGAGCTAAATTTACGT-5'

primer HHV6-A2

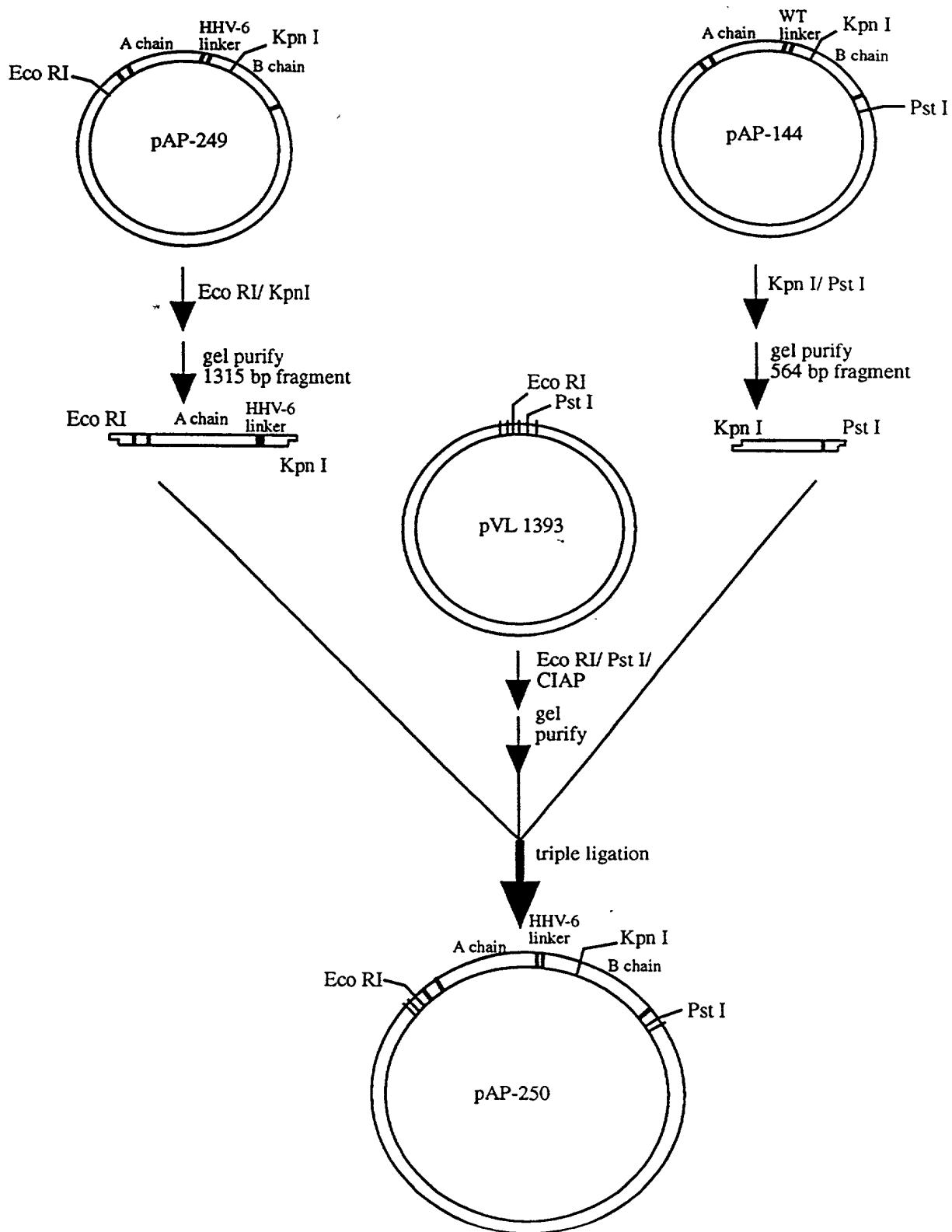
PCR mutagenesis
ligate with pBluescript SK

pAP 249 linker
(HHV-6 variant)

TCCTCGATTTTAAATGCATCGGTGCCAAATTTTAAT
AGAAAGCTAAATTTACGTAGCCACGGTTAAAAATTA

99/254

FIGURE 20C



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100/254

FIGURE 20D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACTGAGCTGATGTGAGACATGATATACCACTGTTGCCAA
AGCAAAATGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCCTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTTA

451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTTCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTCGATTTTAAATGCATCGGTGCCAAATTTTAATGC
AGCAGTGTCAAAAGAAGCTAAAATTTACGTAGCCACGGTTTAAATTTACG

FIGURE 20D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTTCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

102/254

FIGURE 21

Ricin linker (wild type):

A chain- S L L I R P V V P N F N -B chain

pAP-213/pAP-214 linker (Cathepsin B):

A chain- S L L K S R M V P N F N -B chain

pAP-215/pAP-216 linker (MMP-3):

A chain- R P K P Q Q F F G L M N -B chain

pAP-217/pAP-218 linker (MMP-7):

A chain- S L R P L A L W R S F N -B chain

pAP-219/pAP-220 linker (MMP-9):

A chain- S P Q G I A G Q R N F N -B chain

pAP-221/pAP-222 linker (THERMOLYSIN-LIKE MMP):

A chain- D V D E R D V R G F A S F L -B chain

pAP-241/pAP-242 linker (EBV-A):

A chain- S K L V Q A S A S G V N -B chain

pAP-243/pAP-244 linker (EBV-B):

A chain- S S Y L K A S D A P D N -B chain

004440"TSF560

103/254

FIGURE 22A

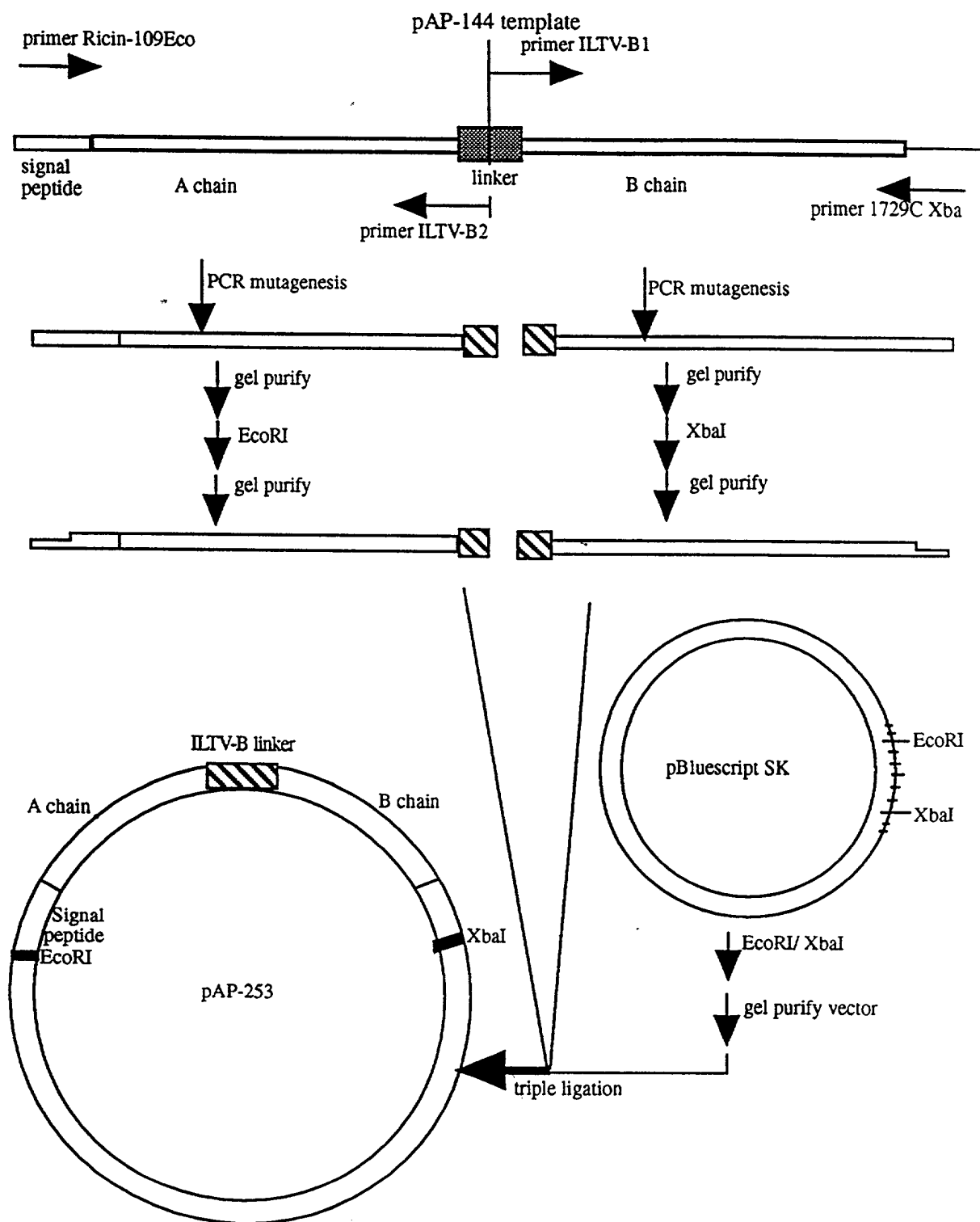


FIGURE 22B

WT preproricin linker

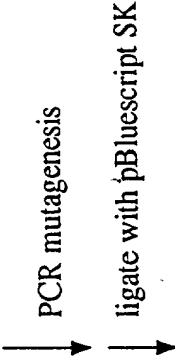
primer ILTV-B1

5' - AATGAGGTAATTACTAATGCTGATGTTTGT -3'

—————TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT—————
—————AGAAACGAATATTCCGGTCACCCACGGTTAAATTA—————

3' - AGCAGTGTCAAAGATTTCATAGATGTCCTCGT-5'

primer ILTV-B2

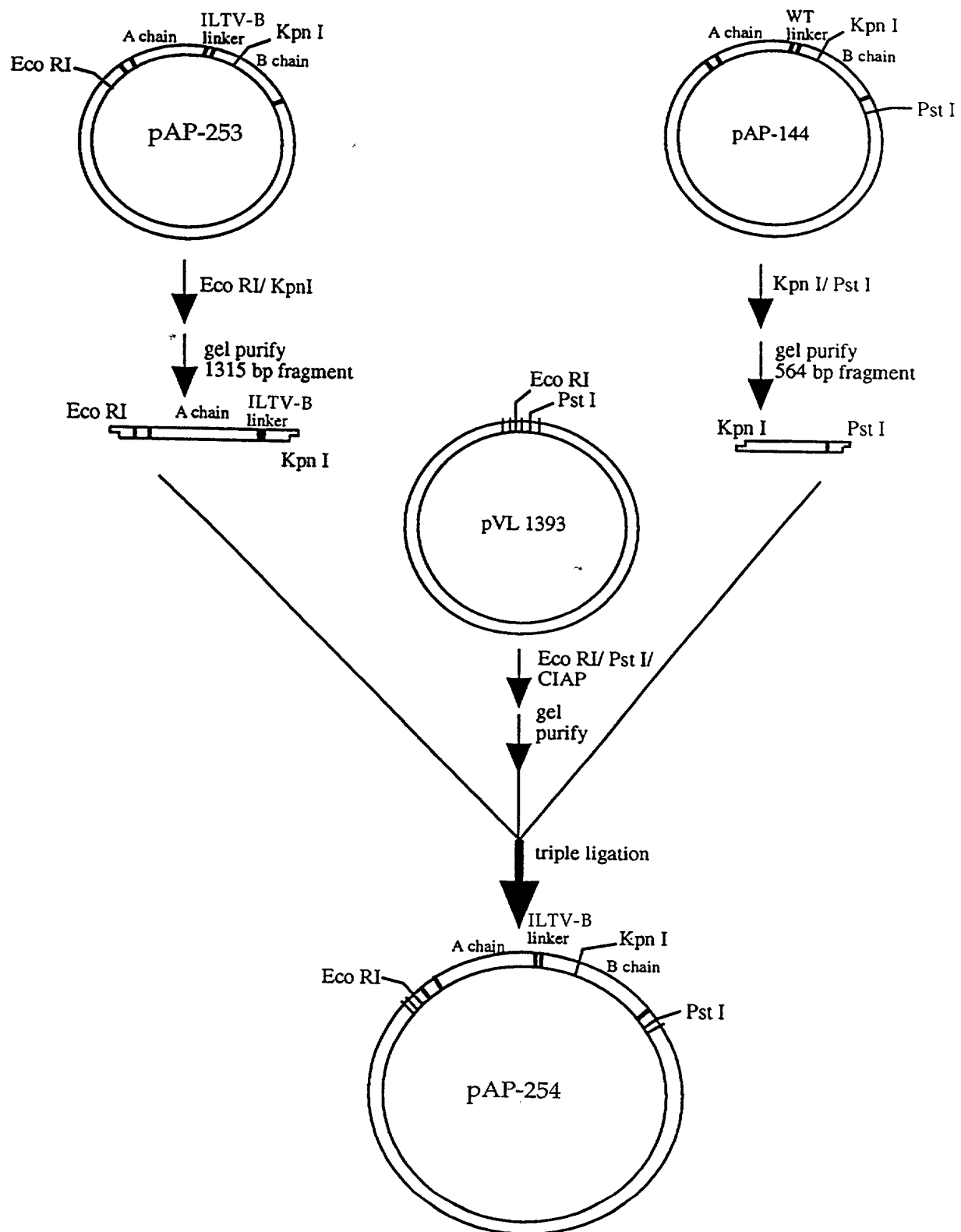


**pAP 253 linker
(ILTV-B variant)**

—————TCTAAGTATCTACAGGCAAAATGAGGTAATTACTAAT—————
—————AGATTCATAGATGTCCTGTTACTCCCATTAATGATTA—————

105/254

FIGURE 22C



106/254

FIGURE 22D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAAC TGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCACGGTTTATTTTAGTTGAACTCTCA
TGTCACAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGA CTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT

901 TCGTCACAGTTTCTAAGTATCTACAGGCAAATGAGGTAATTACTAATGC
AGCAGTGTCAAAAAGATTCATAGATGTCCGTTTACTCCATTAATGATTACG

FIGURE 22D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTTGTCAACCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
 GTTTTGGCTCTATTACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
 AGTTCTTACTACCTTGGTAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAGTGTGTGTGCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTTT
 1801 GGACATTGTAAATTTTGTAAGTGAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

108/254

FIGURE 23A

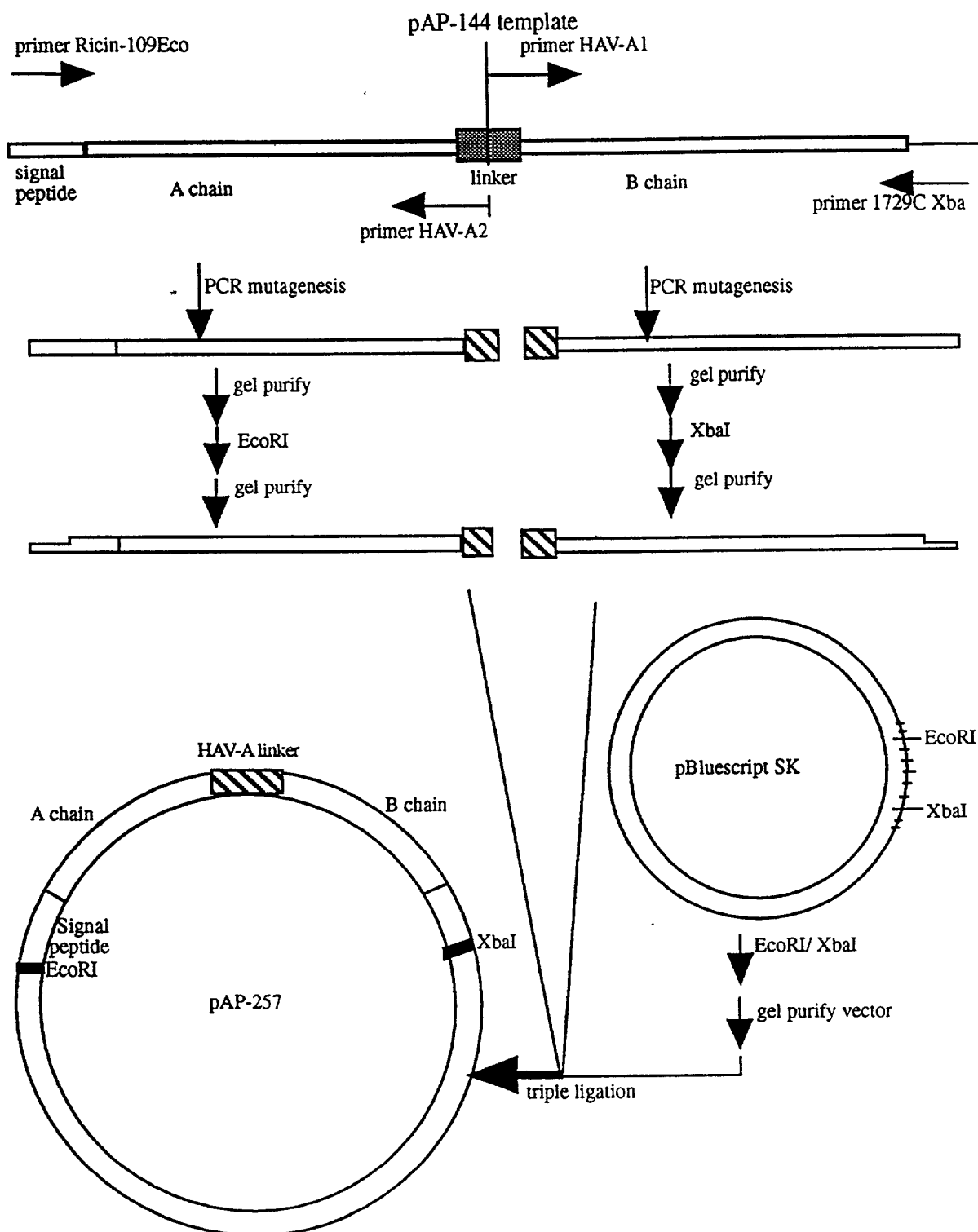


FIGURE 23B

WT preprorcin linker

primer HAV-A1

5' - TCGTTCTCAAATTTGGAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCGGT*
3' - AGCAGTGTCAAAGACTCGAATCTTGGCTT-5'

primer HAV-A2

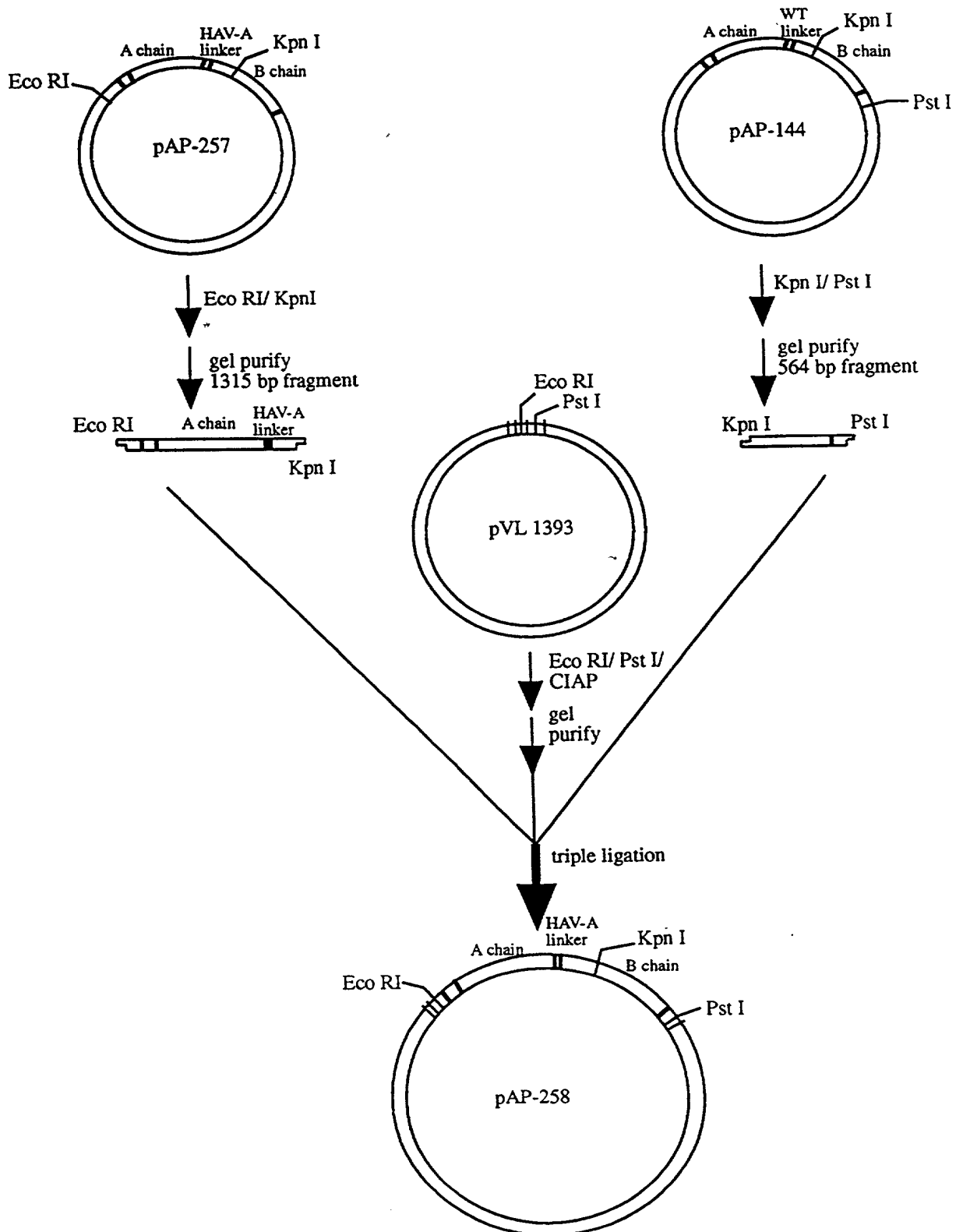
PCR mutagenesis
ligate with pBluescript SK

pAP 257 linker
(HAV-A variant)

TCTGAGCTTAGAACGCAATCGTTCTCAAATTTGGAAT
AGACTCGAATCTTGGCTTAGCAAGAGTTAACCTTA

110/254

FIGURE 23C



111/254

FIGURE 23D

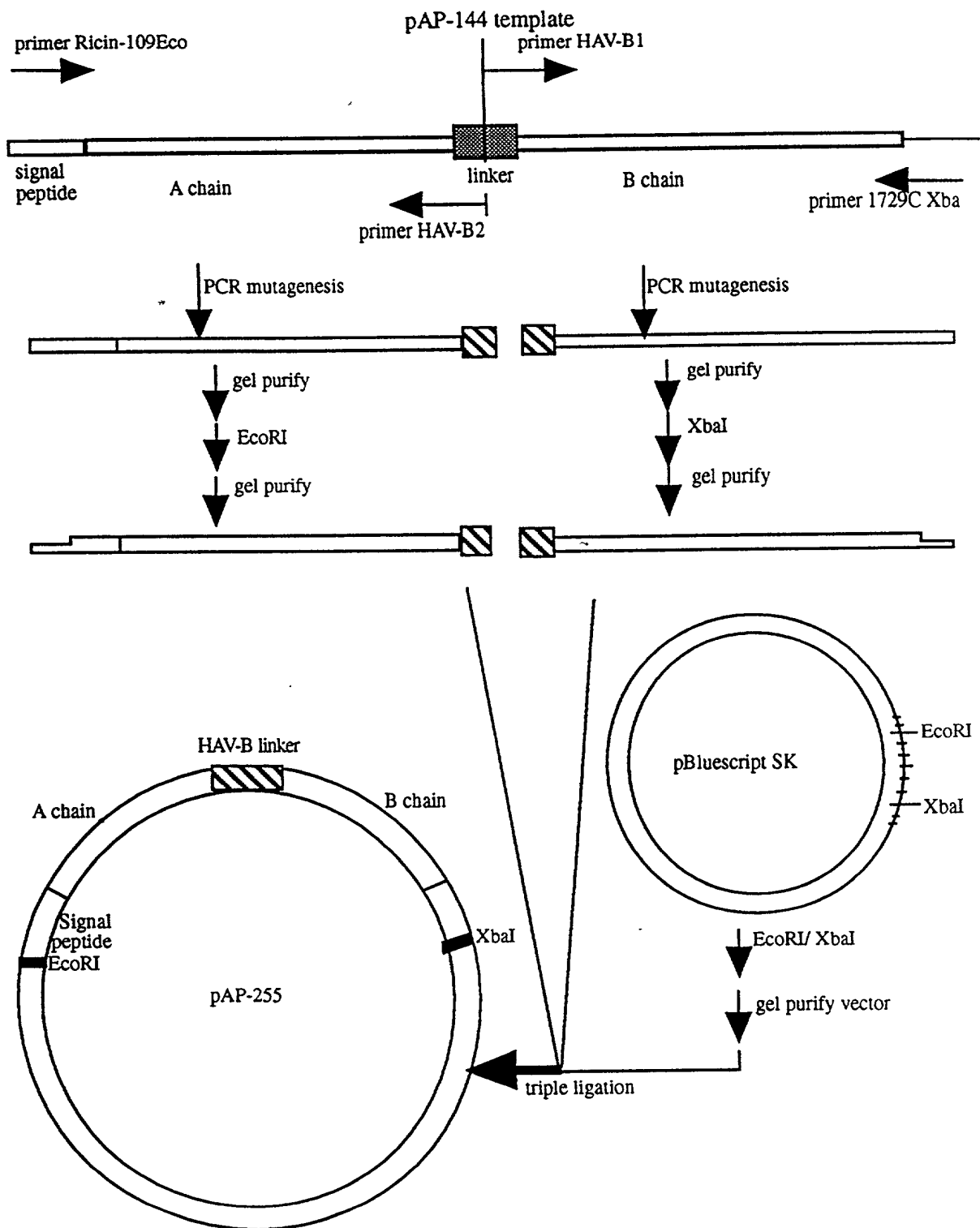
	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTCGCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCACCGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA				
	ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				
701	GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA				
	CTAGACGTGGTCTAGGATCGCATTAATGTGAACCTTTATCAACCCCTCT				
751	CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT				
	GAAAGGTGACGTTAAGTTCTCAGATTGGTTTCTCGGAAACGATCAGGTTA				
801	TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA				
	AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT				
851	TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA				
	ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT				
901	TCGTACAGTTTTCTGAGCTTAGAACGCAATCGTTCTCAAATTGGAATGC				
	AGCAGTGTCAAAAGACTCGAATCTTGCGTTAGCAAGAGTTTAACCTTACG				

FIGURE 23D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
 ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
 1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
 CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
 1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
 GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA
 1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
 CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
 1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
 CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
 1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
 TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
 1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
 GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG
 1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
 AATGTACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
 1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
 TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
 1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
 GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
 1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
 TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
 1501 CAAAACCGAGATAATTGCCCTTACAAGTGATTCTAATATACGGGAACAGT
 GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
 1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
 ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
 1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
 AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCACAATCTA
 1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
 CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
 1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
 ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
 1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
 GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
 1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
 CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
 1851 TGCAG
 ACGTC

113/254

FIGURE 24A



004440"TSF560

FIGURE 24B

WT preprorin linker

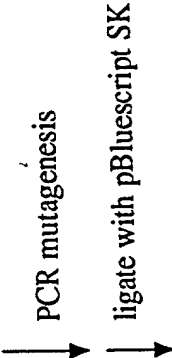
primer HAV-B1

5' - GGGATCGATGATGATAATGCTGATGTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAAATATTCCTGGTCAACACGGTTAAATA

3' - AGCAGTGTCAAAAGACTCGAAACACGCGTT-5'

primer HAV-B2

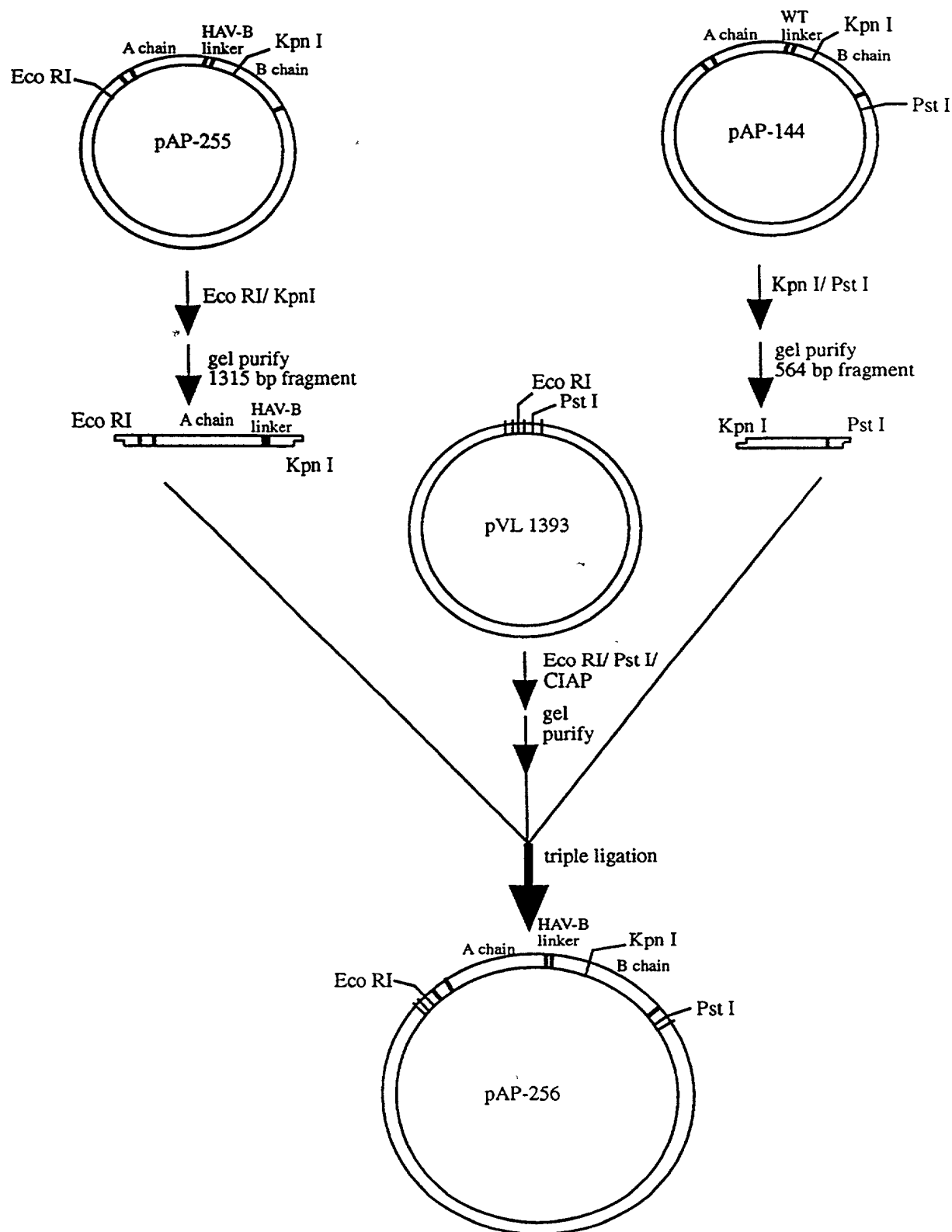


pAP 255 linker
(HAV-B variant)

TCTGAGCTTTGGTCGCAAGGATCGATGATGATAAT
AGACTCGAAACACGCGTTCCCTAGCTACTACTATTA

115/254

FIGURE 24C



004440 044400

116/254

FIGURE 24D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTGAGCTTTGGTCGCAAGGGATCGATGATGATAATGC
AGCAGTGTCAAAAGACTCGAAACCAGCGTTCCCTAGCTACTACTATTACG

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117/254

FIGURE 24D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

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118/254

FIGURE 25A

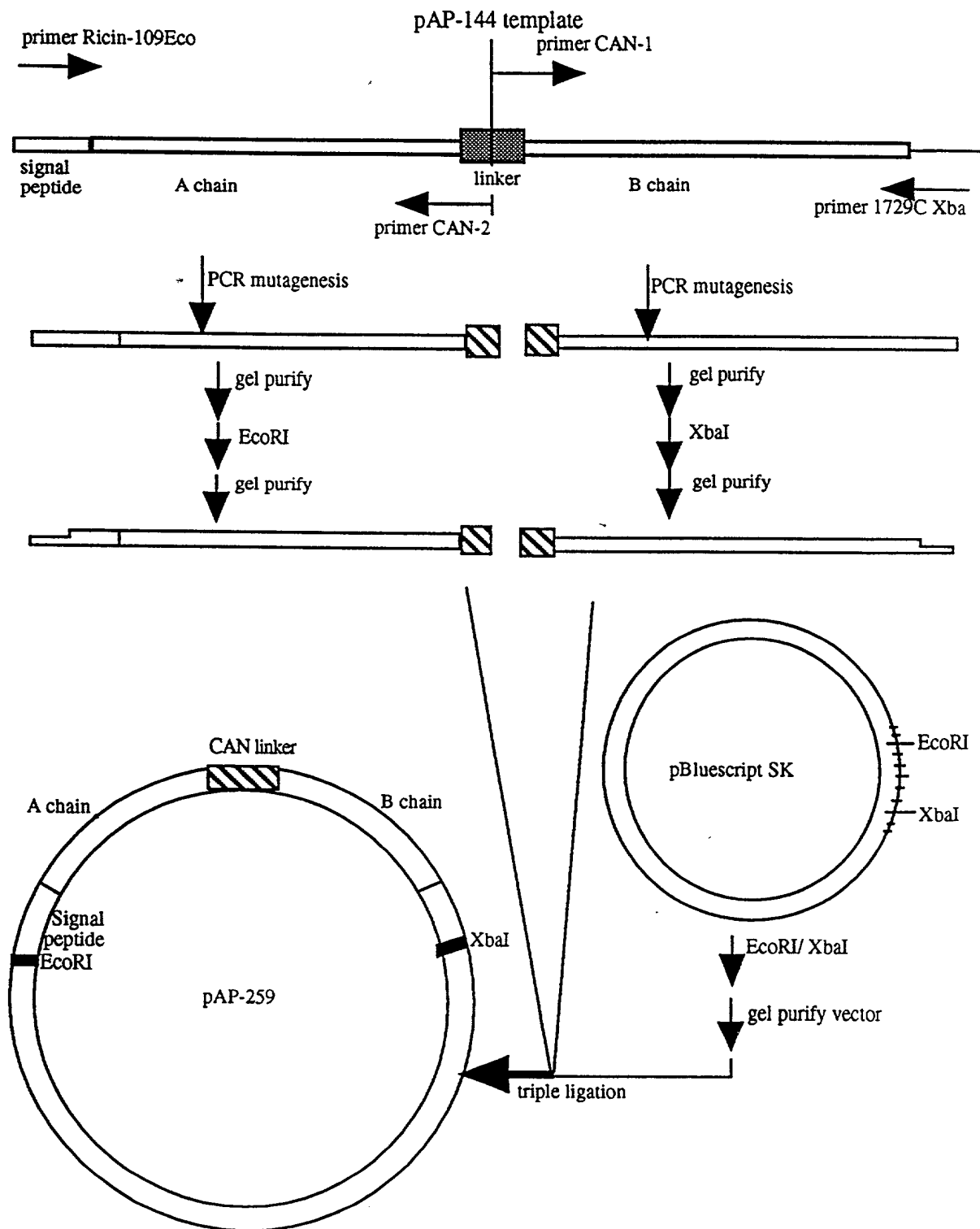


FIGURE 25B

WT preprorin linker

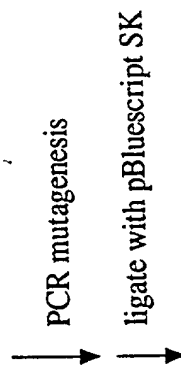
primer CAN-1

5' - TTCAGGCTAAATTTTAATGCTGAT -3'

TCCTTGTCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCTGGTACCCACGGTTAAAAATTA

3' - AGCAGTGTCAAAAGATTCGGACGTTTCAAG-5'

primer CAN-2

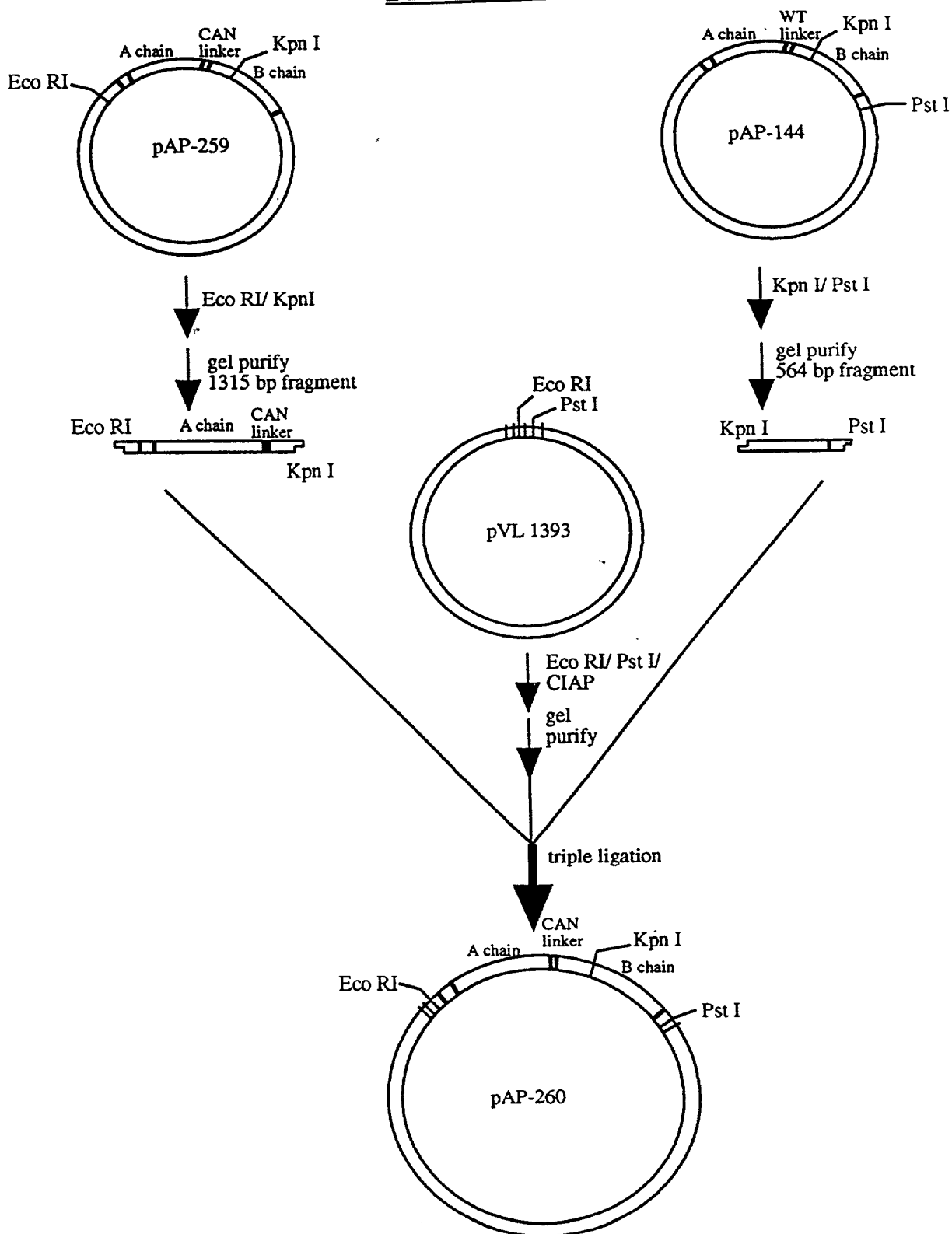


pAP 259 linker (CAN variant)

TCTAAGCCTGCCAAAGTTCCTCAGGCTAAATTTTAAT
AGATTCGGACGTTTCAAGAAGTCCGATTAAAAATTA

120/254

FIGURE 25C



121/254

FIGURE 25D

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTGTTGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCGTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACT
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCTAAGCCTGCAAAGTTCTTCAGGCTAAATTTTAATGC
AGCAGTGTCAAAAGATTTCGGACGTTTCAAGAAGTCCGATTTAAAATTACG

122/254

FIGURE 25D (CONT'D)

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACATAAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTGTGCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTACAAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAAAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGA
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAAATTTAAACATATCACCTAACCAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTTGTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTTATTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCTGTGCTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

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123/254

FIGURE 26

Ricin linker (wild type):

A chain- S L L I R P V V P N F N -B chain

pAP-223/224 linker (MAL-A):

A chain- Q V V Q L Q N Y D E E D -B chain

pAP-225/226 linker (MAL-B):

A chain- L P I F G E S E D N D E -B chain

pAP-227/228 linker (MAL-C):

A chain- Q V V T G E A I S V T M -B chain

pAP-229/230 linker (MAL-D):

A chain- A L E R T F L S F P T N -B chain

pAP-231/pAP-232 linker (MAL-E):

A chain- K F Q D M L N I S Q H Q -B chain

124/254

FIGURE 27

Ricin linker (wild type):

A chain- S L L I R P V V P N F N -B chain

pAP-245/246 linker (CMV-A):

A chain- S G V V N A S C R L A N -B chain

pAP-247/248 linker (CMV-B):

A chain- S S Y V K A S V S P E N -B chain

pAP-233/234 linker (HERPES SIMPLEX-1 A):

A chain- S A L V N A S S A H V N -B chain

pAP-235/236 linker (HERPES SIMPLEX-1 B):

A chain- S T Y L Q A S E K F K N -B chain

pAP-249/250 linker (HUMAN HERPES VIRUS-6):

A chain- S S I L N A S V P N F N -B chain

pAP-237/pAP-238 linker (VZV-A):

A chain- S Q D V N A V E A S S N -B chain

pAP-239/pAP-240 linker (VZV-B):

A chain- S V Y L Q A S T G Y G N -B chain

pAP-253/pAP-254 linker (ILV):

A chain- S K Y L Q A N E V I T N -B chain

pAP-255/pAP-256 linker (HAV-A):

A chain- S E L R T Q S F S N W N -B chain

pAP-257/pAP-258 linker (HAV-B):

A chain- S E L W S Q G I D D D N -B chain

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FIGURE 28

Ricin linker (wild type):

A chain- S L L I R P V V P N F N -B chain

pAP-259/260 linker (CAP-A):

A chain- S K P A K F F R L N F N -B chain

pAP-261/262 linker (CAP-B):

A chain- S K P I E F F R L N F N -B chain

pAP-263/264 linker (CAP-C):

A chain- S K P A E F F A L N F N -B chain

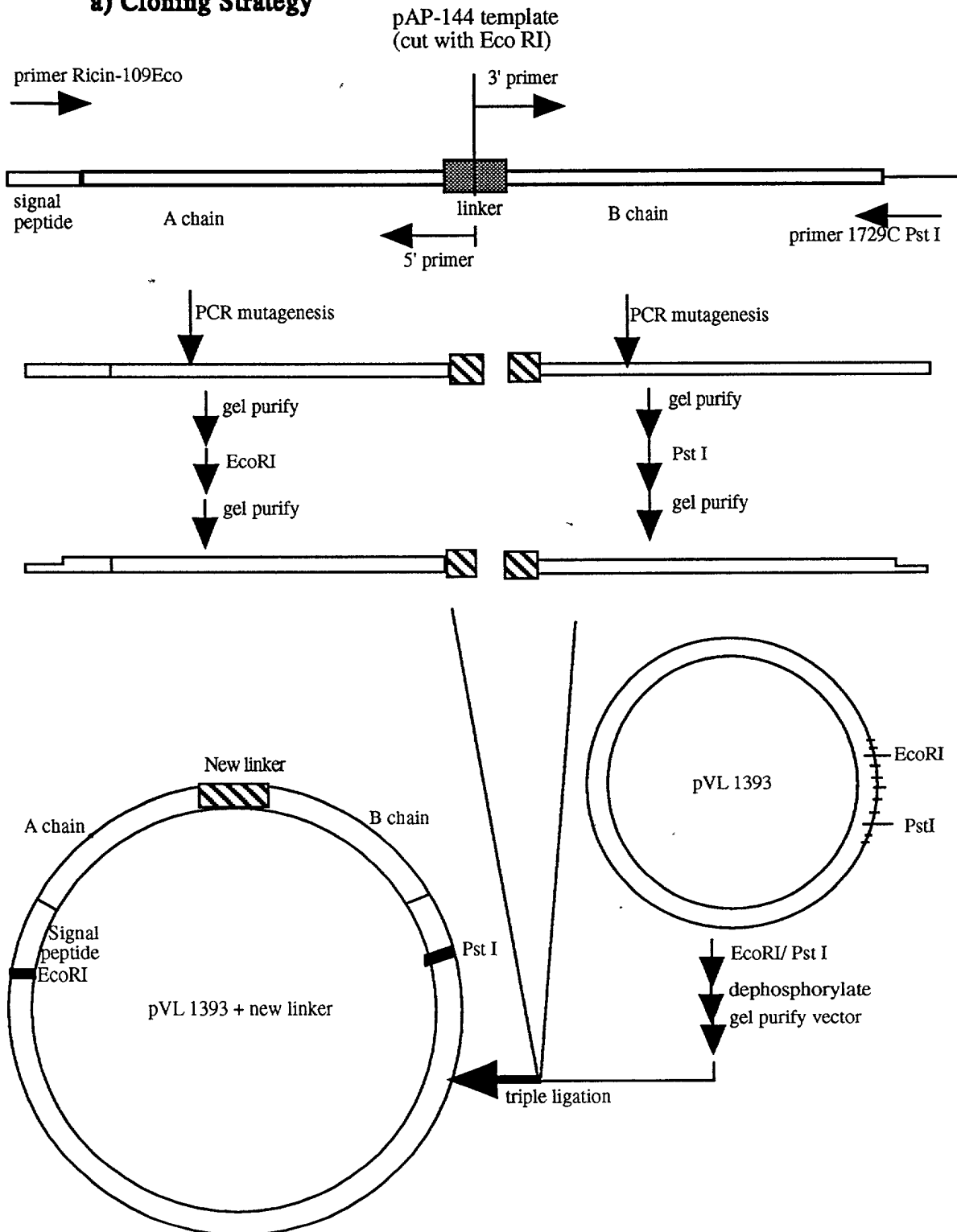
FIGURE 29**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

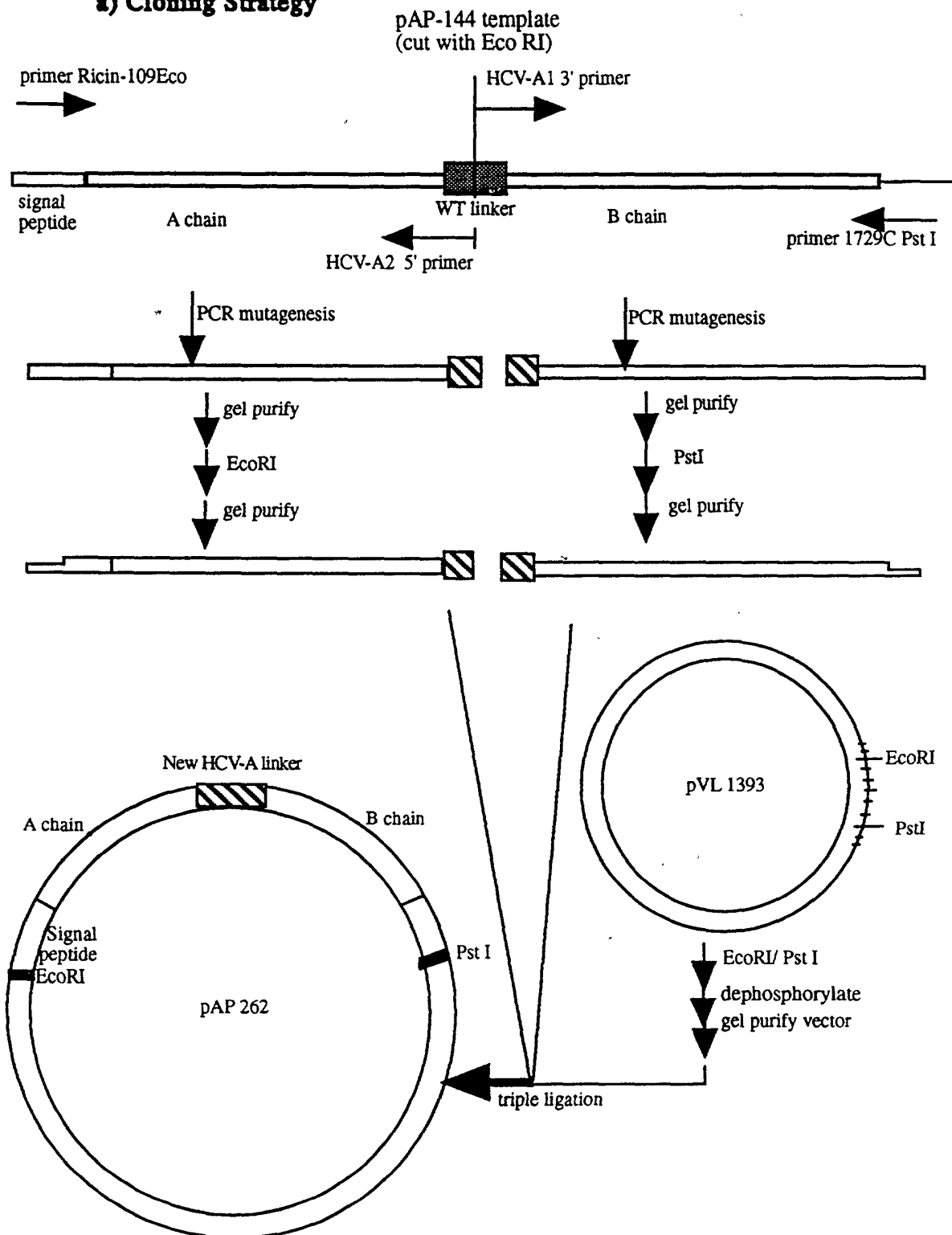
FIGURE 30A**PCR Mutagenesis of Preprorincin Gene to Create An HCV-A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

FIGURE 30B

Sequence of HCV-A Linker Region

WT preprorin linker

primer HCV-A1

5' - TCGACATGGGTTTAAATGCTGATGTT -3'
 ** *
 TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTAAT
 AGAAACGAATATTCGGTCACCCACGGTTTAAATA
 *** *
 3' - GGTAGCAGTGTCAAACTAACCTCCATCACTGT 5'

5' primer HCV-A2

PCR mutagenesis

ligate with pVL1393

pAP 262 linker
 (HCV-A variant)

GATTGGAGGTAGTGACATCGACATGGGTTTAAAT
 CTAAACCTCCATCACTGTAGCTGTACCCAAAAATA

130/254

FIGURE 30C (P2)

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGATTTGGAGGTAGTGACATCGACATGGGTTTTTAATGC
AGCAGTGTCAAACCTCCATCACTGTAGCTGTACCCAAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

131/254

FIGURE 30C (P3)

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP262

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132/254

FIGURE 30D

**-Amino Acid Sequence Comparison of Mutant
Preproricin Linker Region of HCV-A to Wild Type**

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-262 linker: A chain- D L E V V T S T W V F N -B chain
(HCV-A linker)

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133/254

FIGURE 31A

PCR Mutagenesis of Preprorizin Gene to Create An HCV-B Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy

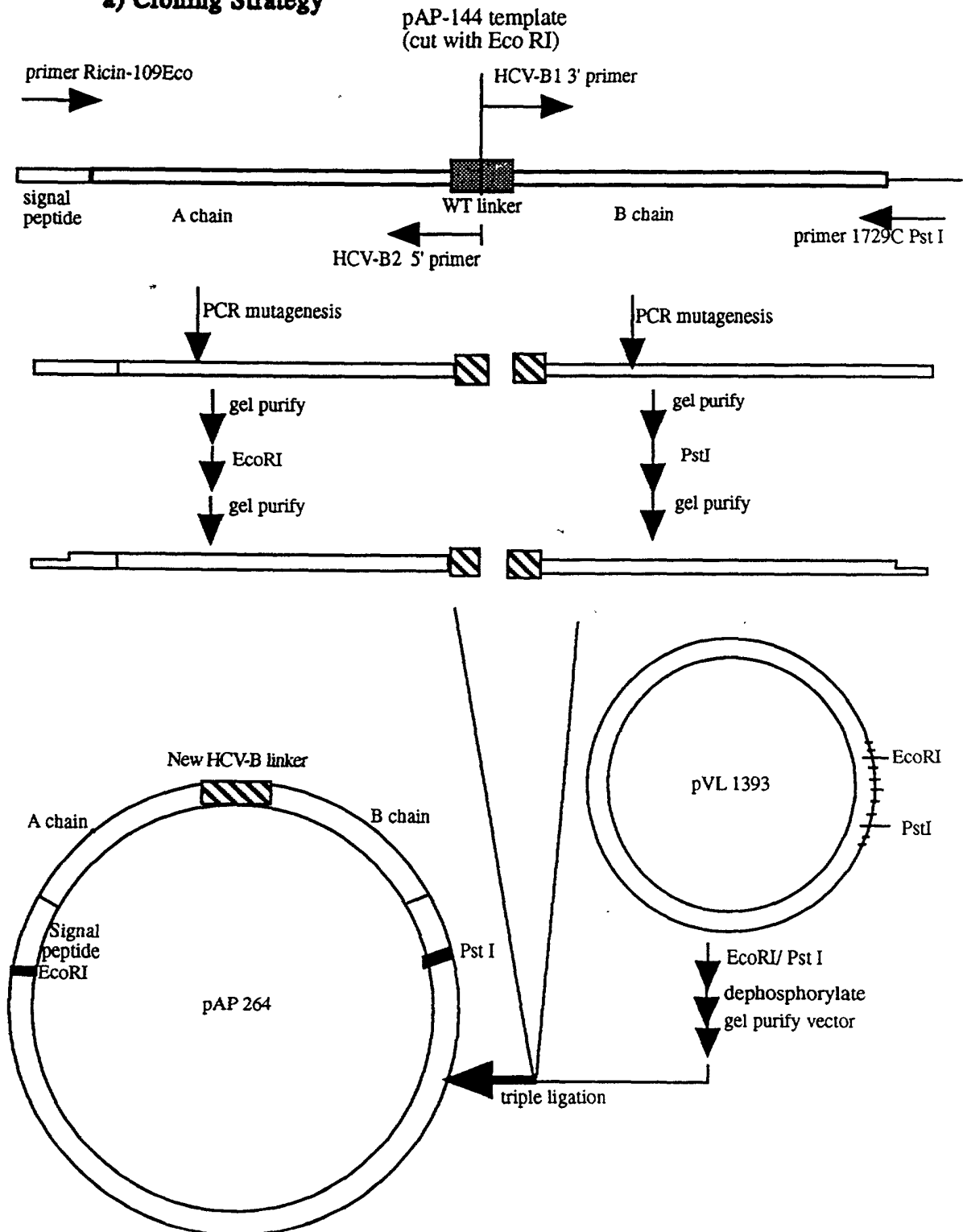


FIGURE 31B

Sequence of HCV-B Linker Region

WT preprorin linker

primer HCV-B1

5' - GCGTCACACCTTTTAAATGCTGATGTT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTAAT
 AGAAACGAATATTCCGGTCACCCAGGTTAAATTA
 3' - GGTAGCAGTGTCAAACTACTACCTTCTCACA -5'

5' primer HCV-B2

PCR mutagenesis
 ligate with pVL1393

pAP 264 linker
(HCV-B variant)

GATGAGATGGAAGAGTGGTGCCACACCTTTTAAAT
 CTACTCTACCTTCTCACACCGCAGTGGGAAAAATTA

135/254

FIGURE 31C (P1)

Sequence of pAP264 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGATATACCACTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
   CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
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136/254

FIGURE 31C (P2)

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGATGAGATGGAAGAGTGTGCGTCACACCTTTTTTAATGC
AGCAGTGTCAAACACTCTACCTTCTCACACGCAGTGTGGAAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCAACATCTA

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FIGURE 31C (P3)

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCA GTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP264

138/254

FIGURE 31D

**-Amino Acid Sequence Comparison of Mutant
Preproricin Linker Region of HCV-B to Wild Type**

Wild type Ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-264 linker: A chain- D E M E E C A S H L F N -B chain
(HCV-B linker)

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0954154.04400

FIGURE 32A

- PCR Mutagenesis of Preprorincin Gene to Create An HCV-C Variant Gene in Baculovirus Transfer Vector, pVL 1393

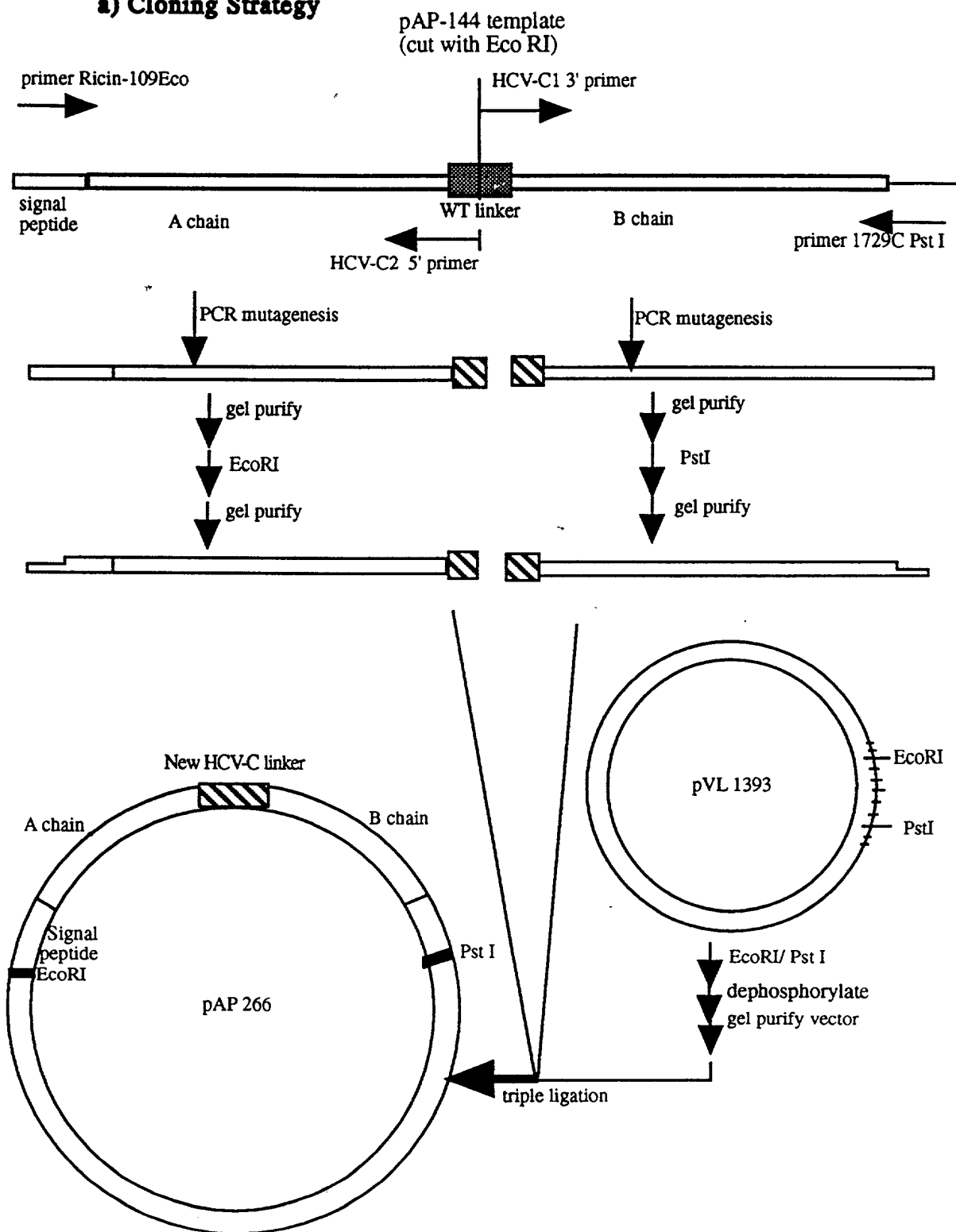
a) Cloning Strategy

FIGURE 32B

Sequence of HCV-C Linker Region

WT preprorin linker

primer HCV-C1

5' - TCGATGTCATATTTTAATGCTGATGTT -3'

TCCTTGCCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGGAATATTCCTGGTCAACACATACAACA-5'

5' primer HCV-C2

PCR mutagenesis

ligate with pVL1393

pAP 266 linker
(HCV-C variant)

GAGGACGTTGTATGTTGTCGATGTCATATTTTAAT
CTCCTGCAACATACAACAAGCTACAGTATAAAATTA

141/254

FIGURE 32C (P1)

Sequence of pAP266 insert

```

          10      20      30      40      50
          |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCTGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
   CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

```

00440" T5T550

142/254

FIGURE 32C (P2)

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGAGGACGTTGTATGTTGTTTCGATGTCATATTTTAATGC
AGCAGTGTCAAACCTGCAACATAACAAGCTACAGTATAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

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143/254

FIGURE 32C (P3)

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP266

00440"EST550

-Amino Acid Sequence Comparison of Mutant Preproricin Linker Region of HCV-C to Wild Type

pAP-266 linker: A chain- E D V V C C S M S Y F N -B chain
(HCV-C linker)

145/254

FIGURE 33A

PCR Mutagenesis of Preproriconin Gene to Create An HCV-D Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy

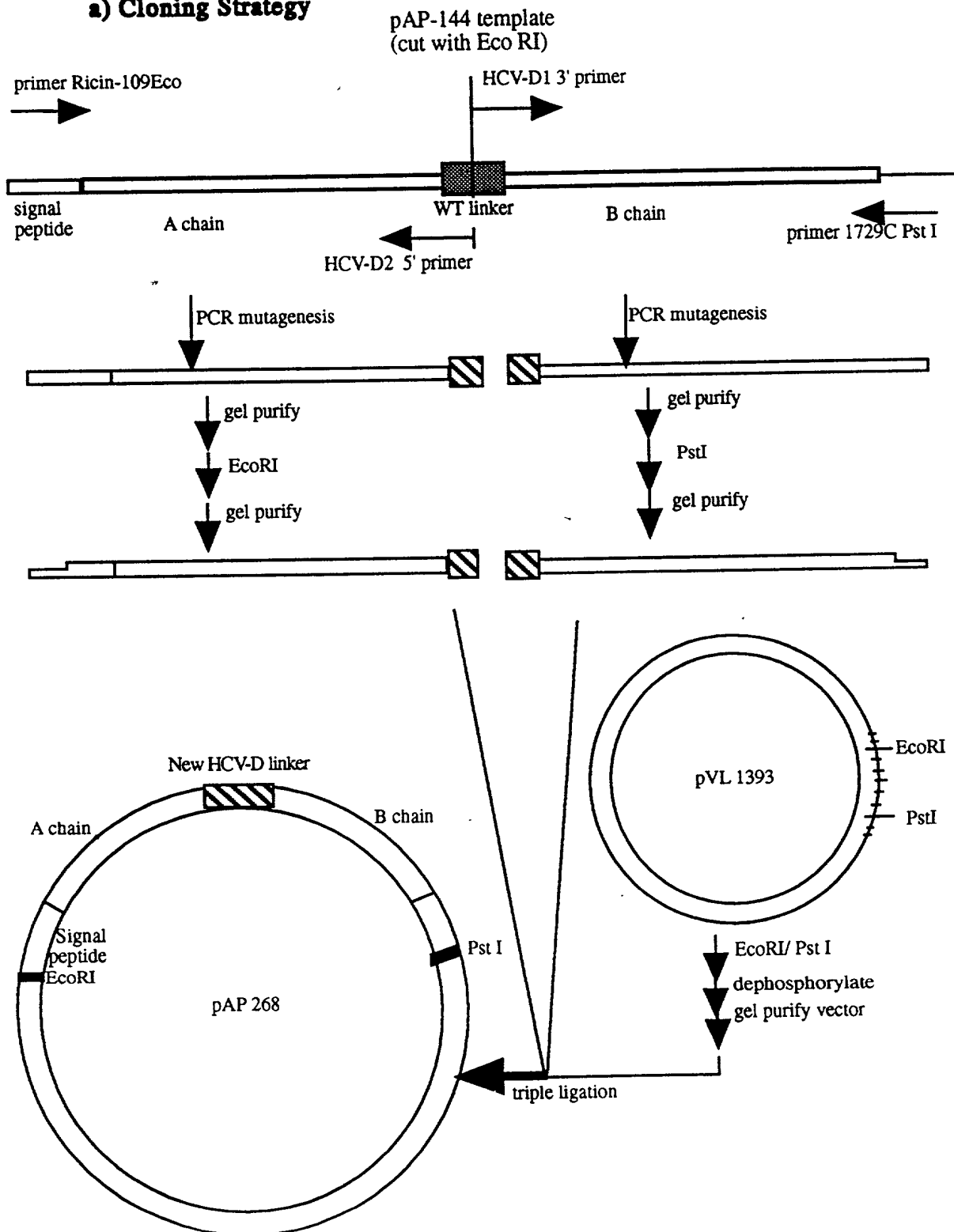


FIGURE 33B

Sequence of HCV-D Linker Region

WT preprorin linker

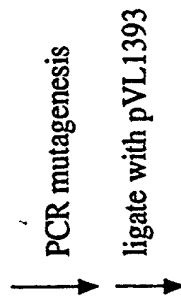
primer HCV-D1

5' - GCGCCAAATAACTGCTTATGCTGATGTTGTATG -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCGGTCACCCAGTTTAAATTA

3' - GGTAGCAGTGTCAAATCCCCACCTCTAACGAT-5'

5' primer HCV-D2



pAP 268 linker
(HCV-D variant)

AAGGGTGGAGATTGCTAGCGCCAAATAACTGCTTAT
TTCCCCACCTCTAACGATCGGGTTATTGACGAATA

147/254

FIGURE 33C (P1)

Sequence of pAP268 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTCGCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCTTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTGCTTC

651 ATCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
   CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
   GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
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148/254

FIGURE 33C (P2)

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTAAAGGGGTGGAGATTGCTAGCGCCAATAACTGCTTATGC
AGCAGTGTCAAATTCCTCCACCTCTAACGATCGCGGTATTGACGAATACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCCTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCTAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTCAAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

00440 15150

FIGURE 33C (P3)

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCA GTGTGTGTGCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCTGTCTGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

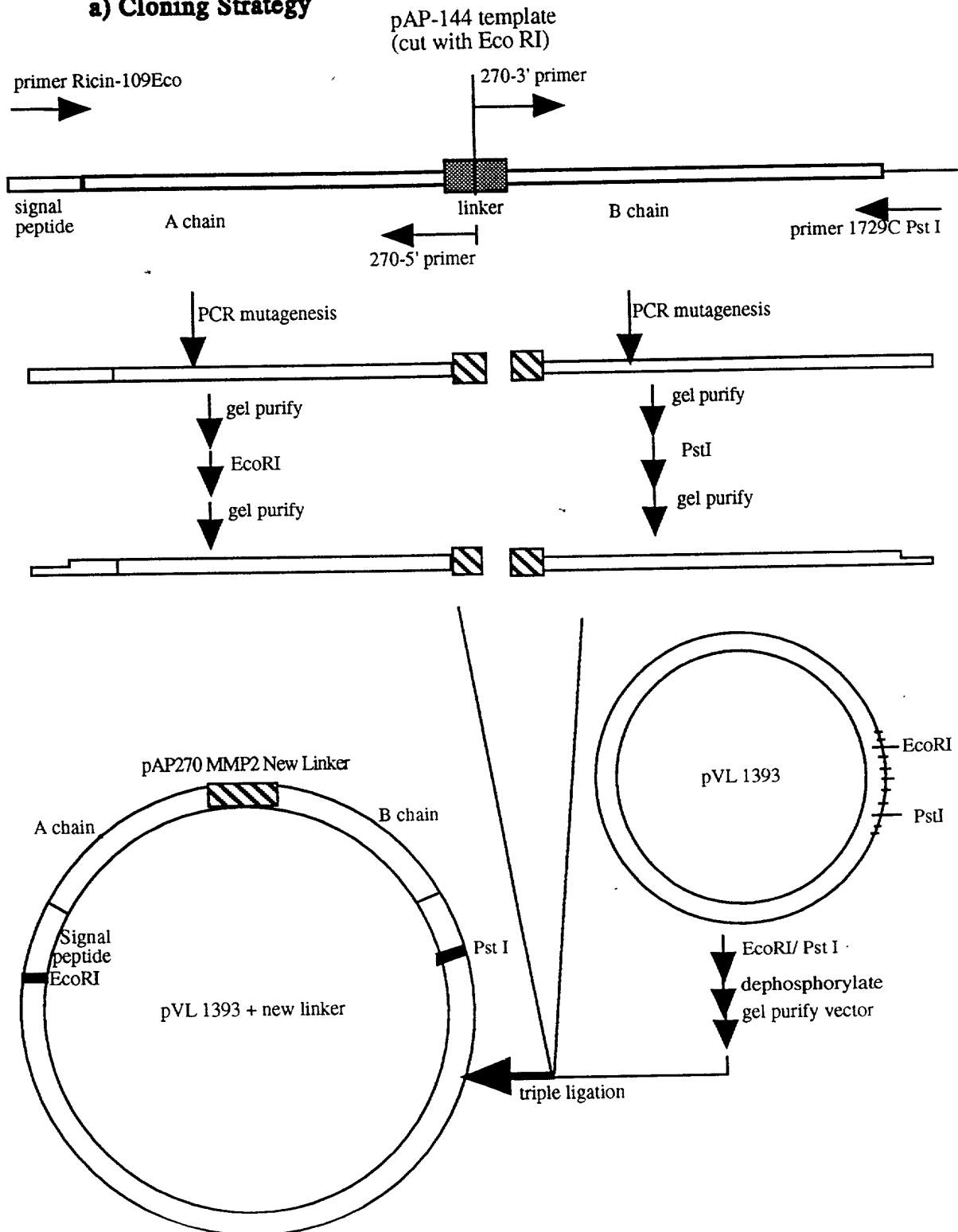
Sequence name: PAP268

-Amino Acid Sequence Comparison of Mutant Preproricin Linker Region of HCV-D to Wild Type

pAP-268 linker: A chain- K G W R L L A P I T A Y -B chain
(HCV-D linker)

FIGURE 34A

PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy

Sequence of MMP-2 Linker Region

primer 270-3'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 270 linker
(MMP-2 variant)**

-----TCTTTTGCCCCCTGGGTTTA|TGGGCTCCTAATTTTAAAT-----
-----AGAAACGGGGACCCAAAT|ACCCGAGGATTAAAATTA-----

153/254

FIGURE 34C (P1)

Sequence of pAP270 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTTGT TTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGT TATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

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154/254

FIGURE 34C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGCCCCTGGGTTTATGGGCTCCTAATTTTAATGC
AGCAGTGTCAAAGAAACGGGGACCCAAATACCCGAGGATTAAATACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCCCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

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FIGURE 34C (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP270

156/254

FIGURE 34D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of MMP-2 to Wild Type**

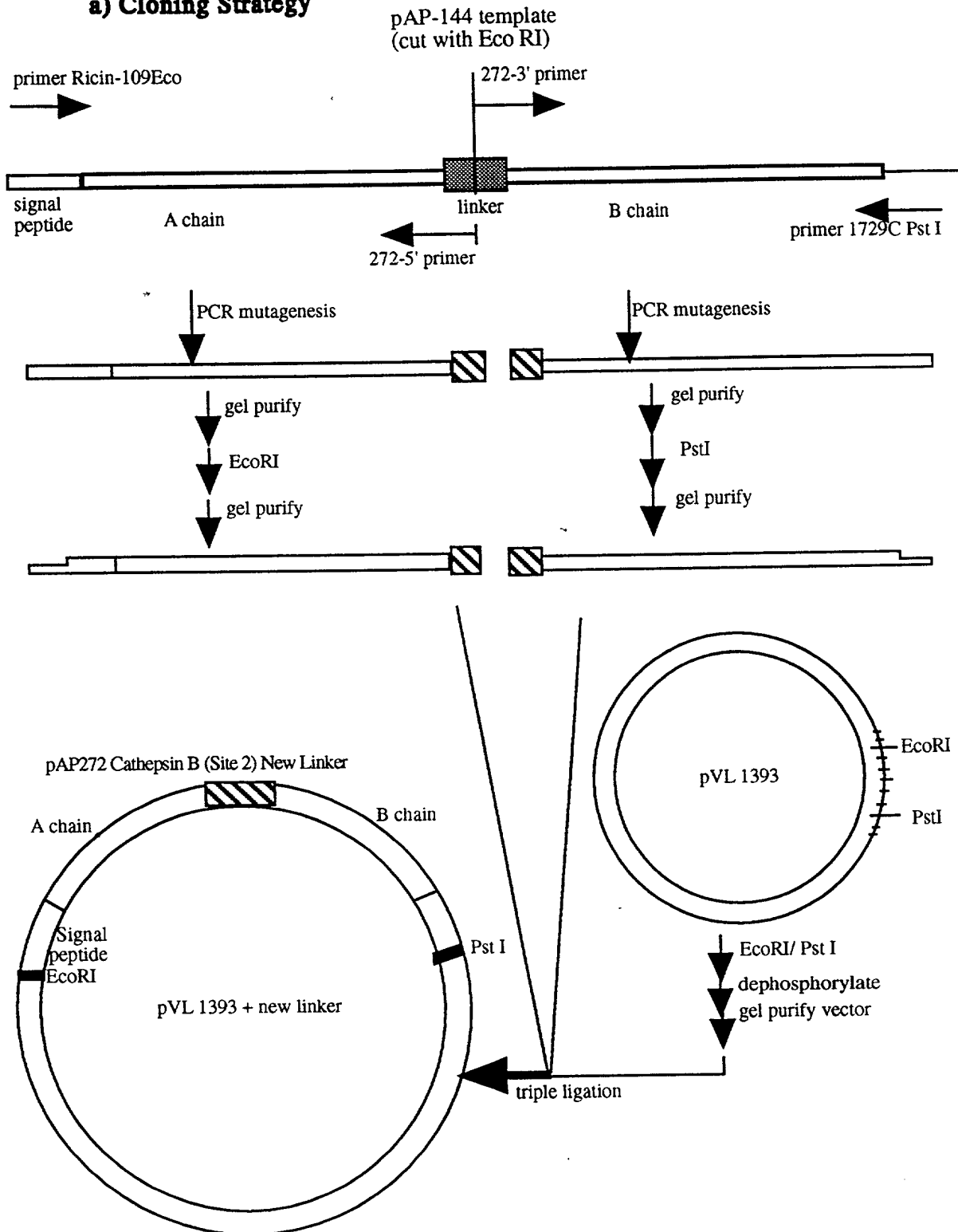
Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-270 (MMP-2) linker: A chain- S L P L G L W A P N F N -B chain

004470 "PST" 004470

FIGURE 35A

PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy

158/254

FIGURE 35B

Sequence of Cathepsin B (Site 2) Linker Region

WT preprocin linker

primer 272-3'
5' - AGGATGCCAAATTTTAATGCTGATGTTTGT -3'
| ** * *
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----
| *****
3' -AGCAGTGTCAAAAGAAACGAATATCGATCT -5'
primer 272-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 272 linker

(Cathepsin B Site 2 variant)

-----TCTTTGCTTATAGCTAGA|AGGATGCCTAATTTTAAT-----
-----AGAAACGAATATCGATCT|TCCTACGGATTAAAATTA-----

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159/254

FIGURE 35C (P1)

Sequence of pAP272 insert

```

          10          20          30          40          50
          |          |          |          |          |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTCAAGTTTAA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

```

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160/254

FIGURE 35C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGCTTATAGCTAGAAGGATGCCTAATTTTAATGC
AGCAGTGTCAAAGAAAGGAATATCGATCTTCCTACGGATTAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTGTTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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161/254

FIGURE 35C (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP272

00440 "T9T9560

162/254

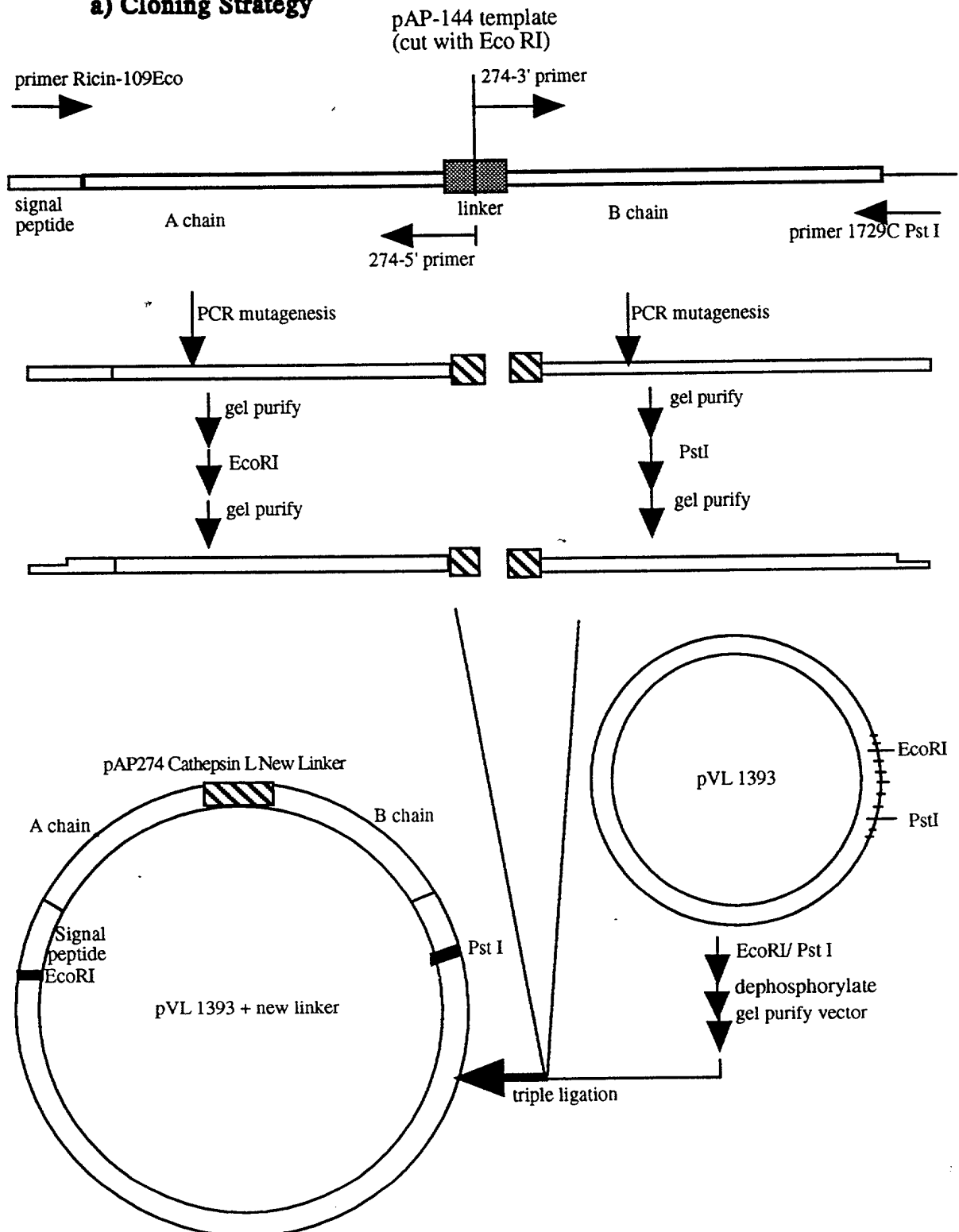
FIGURE 35D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Cathepsin B Site 2 to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-272 (Cathepsin B 2) linker: A chain- S L L I A R R M P N F N -B chain

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FIGURE 36A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

164/254

FIGURE 36B

Sequence of Cathepsin L Linker Region

WT preprocin linker

```

                                primer 274-3'
                                5' - TCATGGGCTAATTTTAATGCTGATGTTTGT -3'
                                | ***** *
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----
                                *** **
3' -AGCAGTGTCAAAAGAAACGAATATAAGGCC -5'
                                primer 274-5'
```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 274 linker (Cathepsin L variant)

```
-----TCTTTGCTTATATTCCGG|TCATGGGCTAATTTTAAT-----
-----AGAAACGAATATAAGGCC|AGTACCCGATTAAAATTA-----
```

165/254

FIGURE 36C (P1)

Sequence of pAP274 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAAGTTTAA

451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAT
   GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

166/254

FIGURE 36C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGCTTATATTCCGGTCATGGGCTAATTTTAATGC
AGCAGTGTCAAAGAAAGGAATATAAGGCCAGTACCCGATTAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACACCTATCTCCTGACATCGTCACTTT

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167/254

FIGURE 36C (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP274

168/254

FIGURE 36D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Cathepsin L to Wild Type**

Wild type ricin linker: , A chain- S L L I R P V V P N F N -B chain

pAP-274 (Cathepsin L)linker: A chain- S L L I F R S W A N F N -B chain

00440" 454360

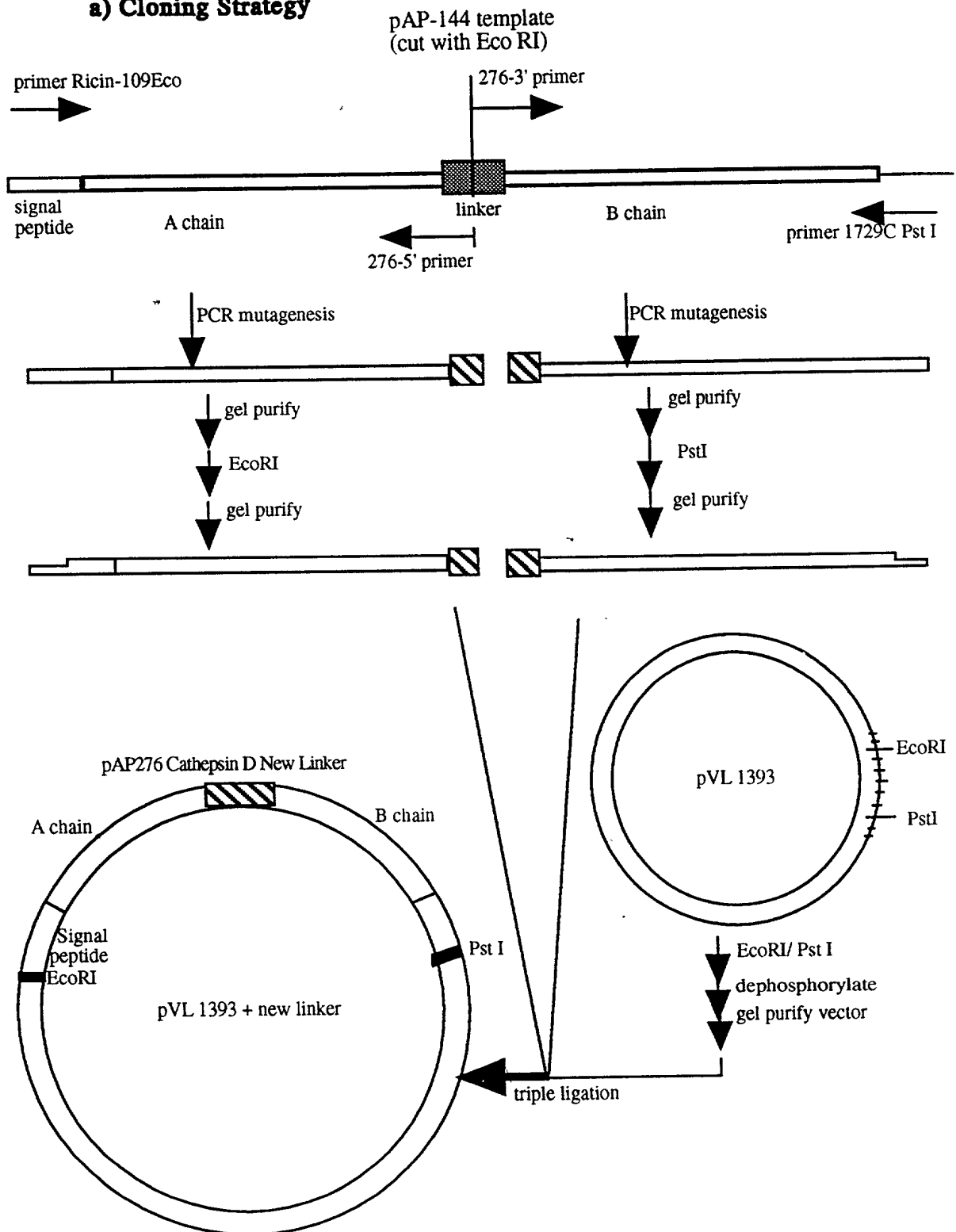
FIGURE 37A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

FIGURE 37B

Sequence of Cathepsin D Linker Region

WT preprocin linker

```

          primer 276-3'
          5'- ACTGTTATTGTTATCACCGCTGATGTTTGT -3'
                    | ***      * * *
-----|TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----|AGATAACGAATATTCCGG|CACCATGGTTTAAAATTA-----
          **** * * * *
3'-AGCAGTGTCAAAGACCACAACAGTAGCGA -5'
          primer 276-5'

```

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

**pAP 276 linker
(Cathepsin D variant)**

-----TCTGGTGTGTGTCATCGCT|ACTGTTATTGTTATCACC-----
-----AGACCACAACAGTAGCGA|TGACAATAACAATAGTGG-----

171/254

FIGURE 37C (P1)

Sequence of pAP276 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATAACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTAA
451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACGTAAGTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

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172/254

FIGURE 37C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTGGTGTTCATCGCTACTGTTATTGTTATCACCGC
AGCAĜTGTCAAAAGACCACAACAGTAGCGATGACAATAACAATAGTGGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACCTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCĤTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTCTGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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173/254

FIGURE 37C (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP276

174/254

FIGURE 37D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Cathepsin D to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-276 (Cathepsin D) linker: A chain- S G V V I A T V I V I T -B chain

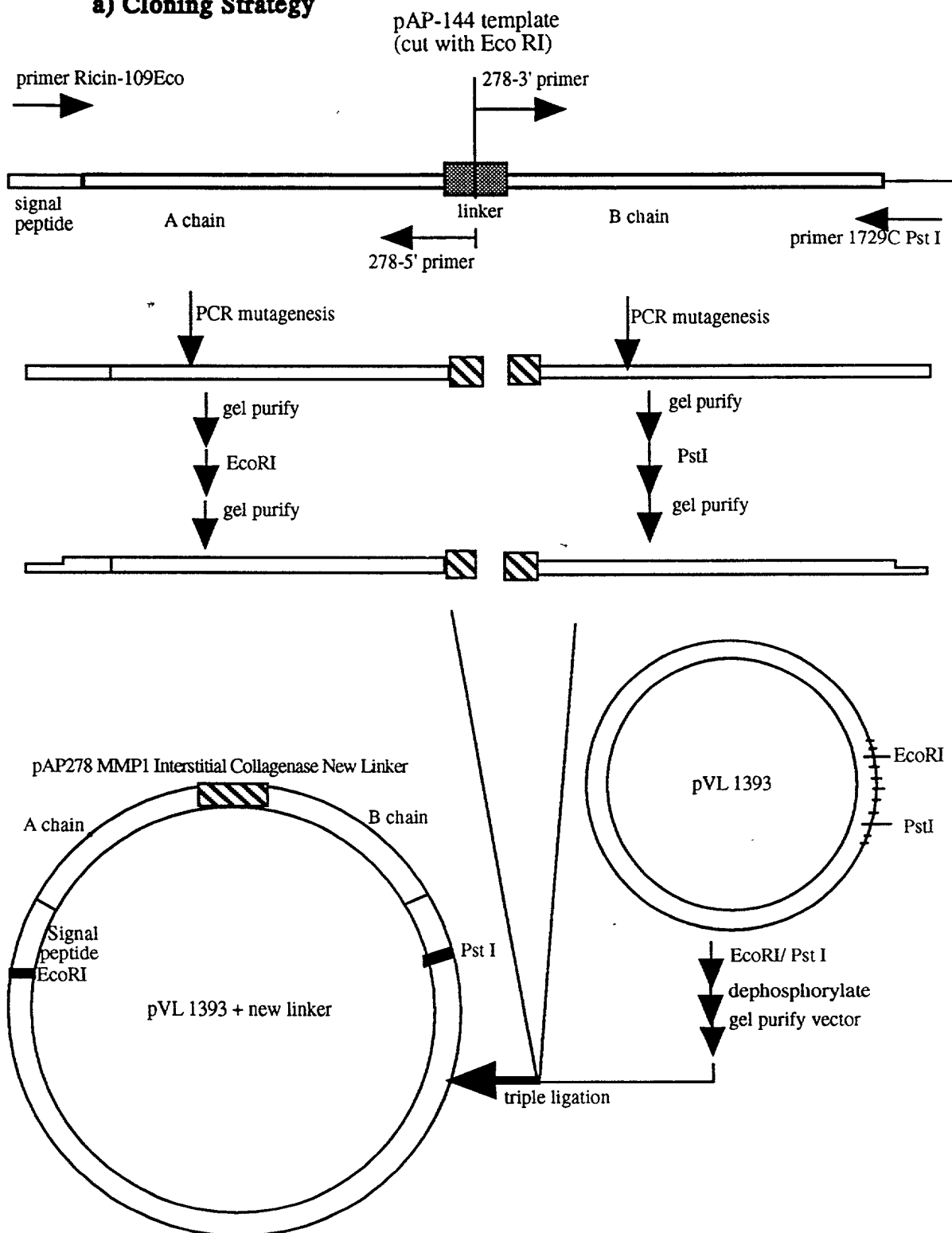
00440"TSF560

175/254

FIGURE 38A

PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy



176/254

FIGURE 38B

Sequence of MMP-1 (Interstitial collagenase) Linker Region

WT preprocin linker

primer 278-3'
5' - ATTTGGGGACAGTTTAATGCTGATGTTTGT -3'
* * * * *
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----
** * * * * *
3' -AGCAGTGTCAAAAGAAACCCAGGAGTTCCG -5'
primer 278-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 278 linker (MMP-1 variant)

-----TCTTTGGGTCCTCAAGGC|ATTTGGGGACAGTTTAAT-----
-----AGAAACCCAGGAGTTCCG|TAAACCCCTGTCAAATTA-----

00440" F5F550

177/254

FIGURE 38C (P1)

Sequence of pAP278 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGCTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCACTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

178/254

FIGURE 38C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGGGTCTCAAGGCATTTGGGGACAGTTTAATGC
AGCAGTGTCAAAGAAACGCAGGAGTTCCGTAAACCCCTGTCAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

179/254

FIGURE 38C (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP278

00440 "PAP278"

180/254

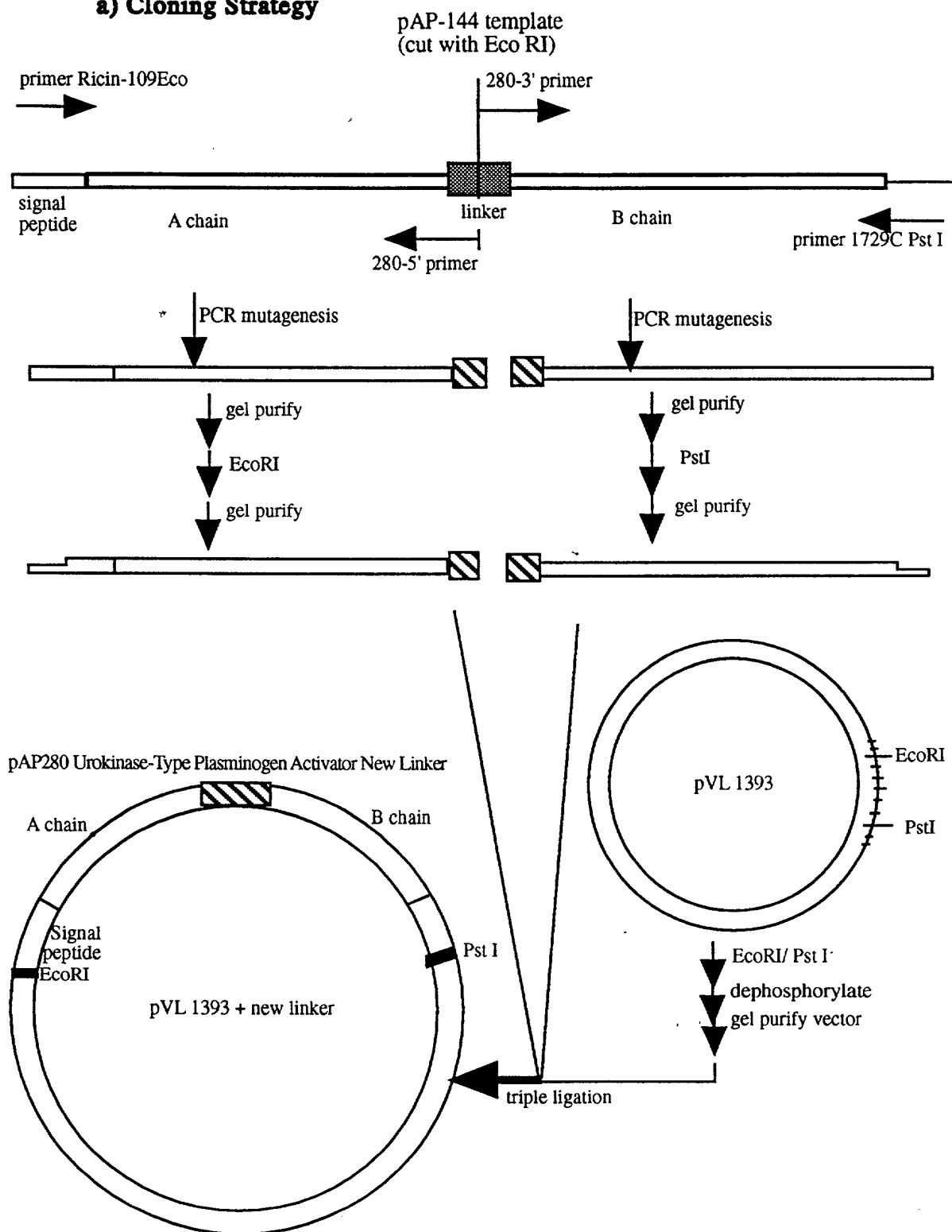
FIGURE 38D

Figure 38. d) Amino acid sequence Comparison of Mutant Preproricin Linker region of MMP-1 (Interstitial collagenase) to Wild Type

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-278 (MMP-1) linker: A chain- S L G P Q G I W G Q F N -B chain

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FIGURE 39A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

182/254

FIGURE 39B

Sequence of Urokinase-Type Plasminogen Activator Linker Region

WT preprocin linker

primer 280-3'
5'- GTTGTCGGTGGCTCTGTAGCTGATGTTTGT -3'
* * * * *
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAATTA-----

3'-AGCAGTGTCAAATTTTTTTAGGGGACCTTCT -5'
primer 280-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 280 linker (uPA variant)

-----AAAAAATCCCCTGGAAGA|GTTGTCGGTGGCTCTGTA-----
-----TTTTTTAGGGGACCTTCT|CAACAGCCACCGAGACAT-----

183/254

FIGURE 39C (P1)

Sequence of pAP280 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

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184/254

FIGURE 39C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCAATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTAAAAAATCCCCTGGAAGAGTTGTGGTGGCTCTGTAGC
AGCAGTGTCAAATTTTTTAGGGGACCTTCTCAACAGCCACCGAGACATCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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[illegible][illegible][illegible][illegible]

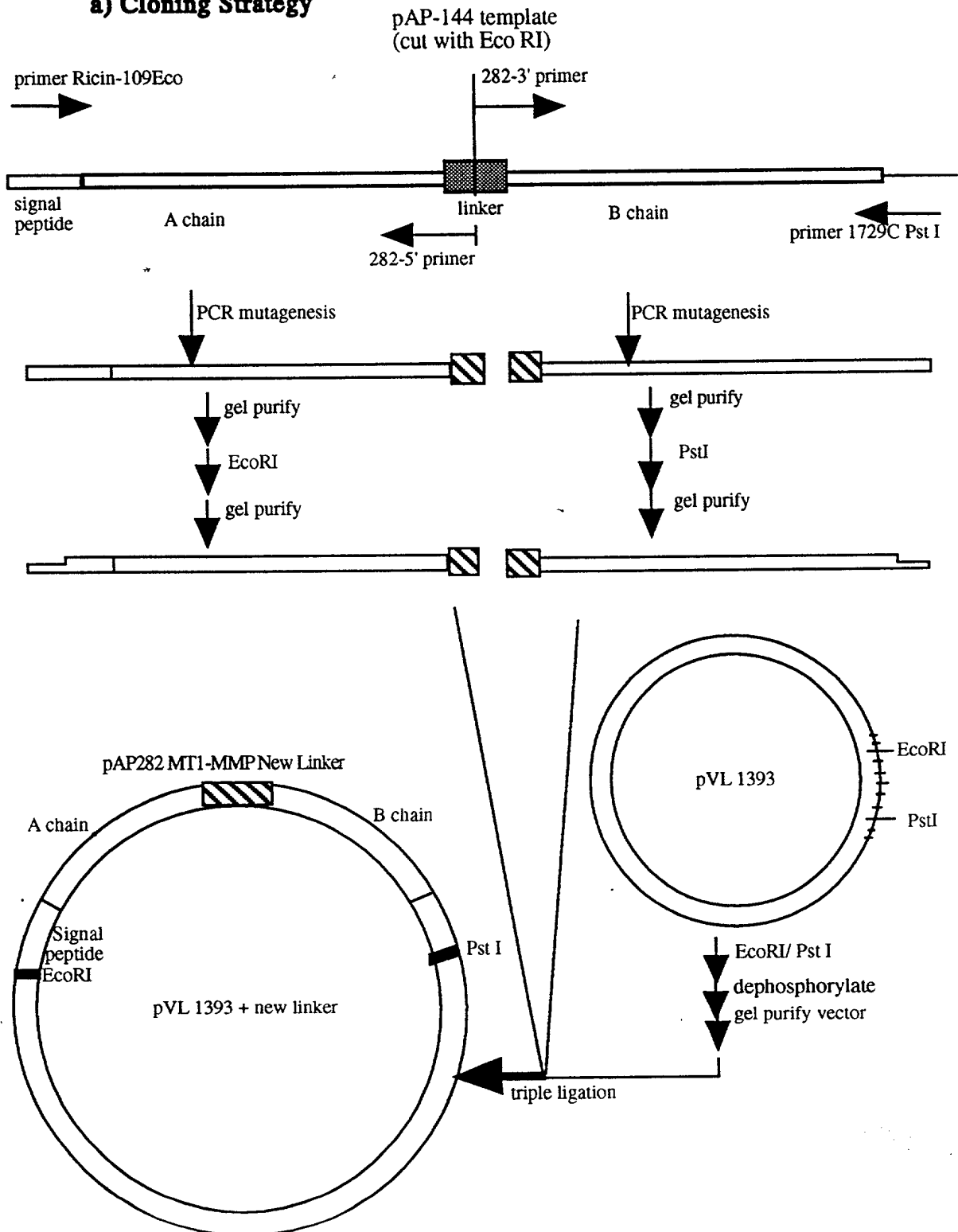
186/254

FIGURE 39D

Figure 39. d) Amino acid sequence Comparison of Mutant Preproricin Linker region of Urokinase-Type Plasminogen Activator to Wild Type

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-280 (uPA) linker: A chain- K K S P G R V V G G S V-B chain

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FIGURE 40A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

-----CCCCAAGGACTCCTAGGG|GCTCCTGGTATTCTTGGC-----
-----GGGGTTTCCTGAGGATCCC|CGAGGACCATAAGAACCG-----

189/254

FIGURE 40C (P1)

Sequence of pAP282 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTIONAAGTTTAA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACCT
   GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

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190/254

FIGURE 40C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTCCCCAAGGACTCCTAGGGGCTCCTGGTATTCTTGGCGC
AGCAGTGTCAAAGGGGTTCTGAGGATCCCCGAGGACCATAAGAACCGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

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FIGURE 40C (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTACCCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CA[^]AAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTTATTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTGCTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP282

Amino acid sequence Comparison of Mutant Preproricin Linker region of MT-MMP to Wild Type

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-282 (MT-MMP) linker: A chain- P Q G L L G A P G I L G-B chain

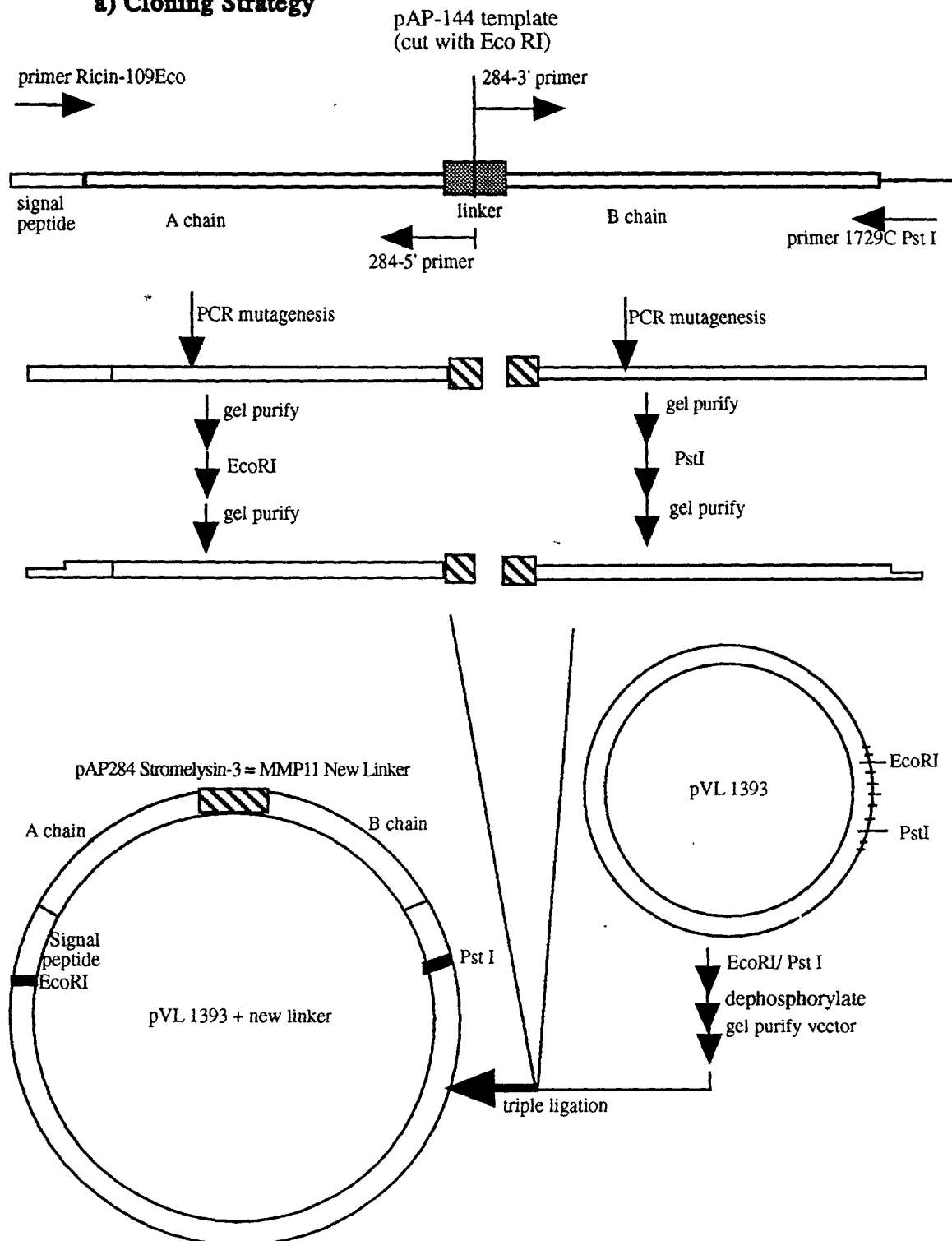
FIGURE 41A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

FIGURE 41B

Sequence of MMP-11 (Stromelysin-3) Linker Region

WT preprocin linker

primer 284-3'
 5' - ATGGGAAGAGGCCCATGCTCGTTTAGTTTCATGTCGAAAGAGCCTCACACTGCTGATGTTTGTATGGAT-3'
 -----TCTTTTGCTTATAAGGCCA | GTGGTACCACAAATTTTAAT-----
 -----AGAAACGAATATTCGGGT | CACCATGGTTTAAATTA-----

3'-GGTGGTAGCAGTGTCAGGGGCTCCCAAATTTCTCACCCTAAAAATACTTAGACTGCAG -5'
 primer 284-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 284 linker

(MMP-11 variant)

---CACGGCCCCGAGGGTTTAAGAGTGGGATTTTATGAATCTGACGTC | ATGGGAAGAGGCCCATGCTCGTTTAGTTTCATGTCGAAAGAGCCTCACACT---
 ---GTGCGGGGCTCCCAAATTTCTCACCCTAAAAATACTTAGACTGCAG | TACCCCTTCTCCGGTACGAGCAAAATCAAGTACAGCAACTCGGAGTGTGA---

195/254

FIGURE 41C (P1)

Sequence of pAP284 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCACTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
```

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196/254

FIGURE 41C (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTT
AGCAGTGTCAAA

Linker Sequence:

CACGGCCCCGAGGGTTTAAGAGTGGGATTTTATGAATCTGACGTCATGGG
GTGCCGGGGCTCCCAAATTCTCACCTAAAATACTTAGACTGCAGTACCC

AAGAGGCCATGCTCGTTTAGTTTCATGTGGAAGAGCCTCACACT
TTCTCCGGTACGAGCAAATCAAGTACAGCAACTCGGAGTGTGA

949 GC
CG

951 TGATGTTTG TATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTT CAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

197/254

FIGURE 41C (P3)

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACCAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCTCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

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198/254

FIGURE 41D

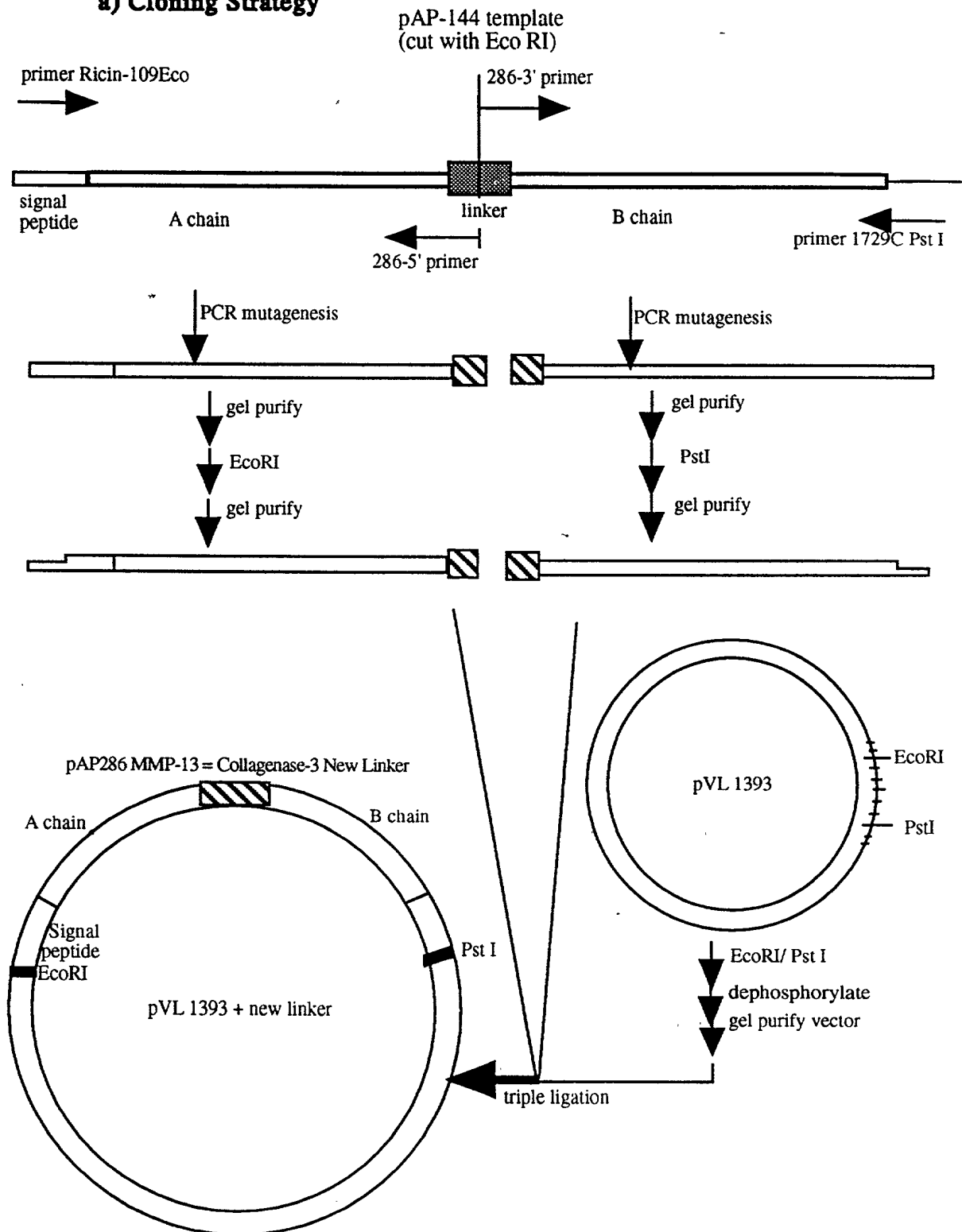
**Amino acid sequence Comparison of Mutant Preproricin Linker
region of MMP-11 (Stromelysin-3) to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-284 (MMP-11) linker:

A chain- H G P E G L R V G F Y E S D V M G R G H A R L V H V E E P H T -B chain

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FIGURE 42A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

200/254

FIGURE 42B

Sequence of MMP-13 = Collagenase-3 Linker Region

WT preprocin linker

```
primer 286-3'
5'- GGTCAACGAGGCATTGTCGCTGATGTTTGT -3'
      **** * **** *
-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAATTA-----
      *****
3'-AGCAGTGTCAAACCTGGAGTCCCCGAACGA -5'
primer 286-5'
```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 286 linker (MMP-13 variant)

```
-----GGACCTCAGGGGCTTGCT|GGTCAACGAGGCATTGTC-----
-----CCTGGAGTCCCCGAACGA|CCAGTTGCTCCGTAAACAG-----
```

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201/254

FIGURE 42C (P1)

Sequence of pAP286 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATAACAGTGTGCGCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTGTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAACTATCTGAAGTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
```

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202/254

FIGURE 42C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGACCTCAGGGGCTTGCTGGTCAACGAGGCATTGTTCG
AGCAGTGTCAAACCTGGAGTCCCCGAACGACCAGTTGCTCCGTAACAGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTGTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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203/254

FIGURE 42C (P3)

GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
1551 TGTAAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTGCTACCTACA
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA
1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

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204/254

FIGURE 42D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of MMP-13 (Collagenase-3) to Wild Type**

Wild type ricin linker:	A chain- S L L I R P V V P N F N -B chain
pAP-286 (MMP-13) linker:	A chain- G P Q G L A G Q R G I V -B chain

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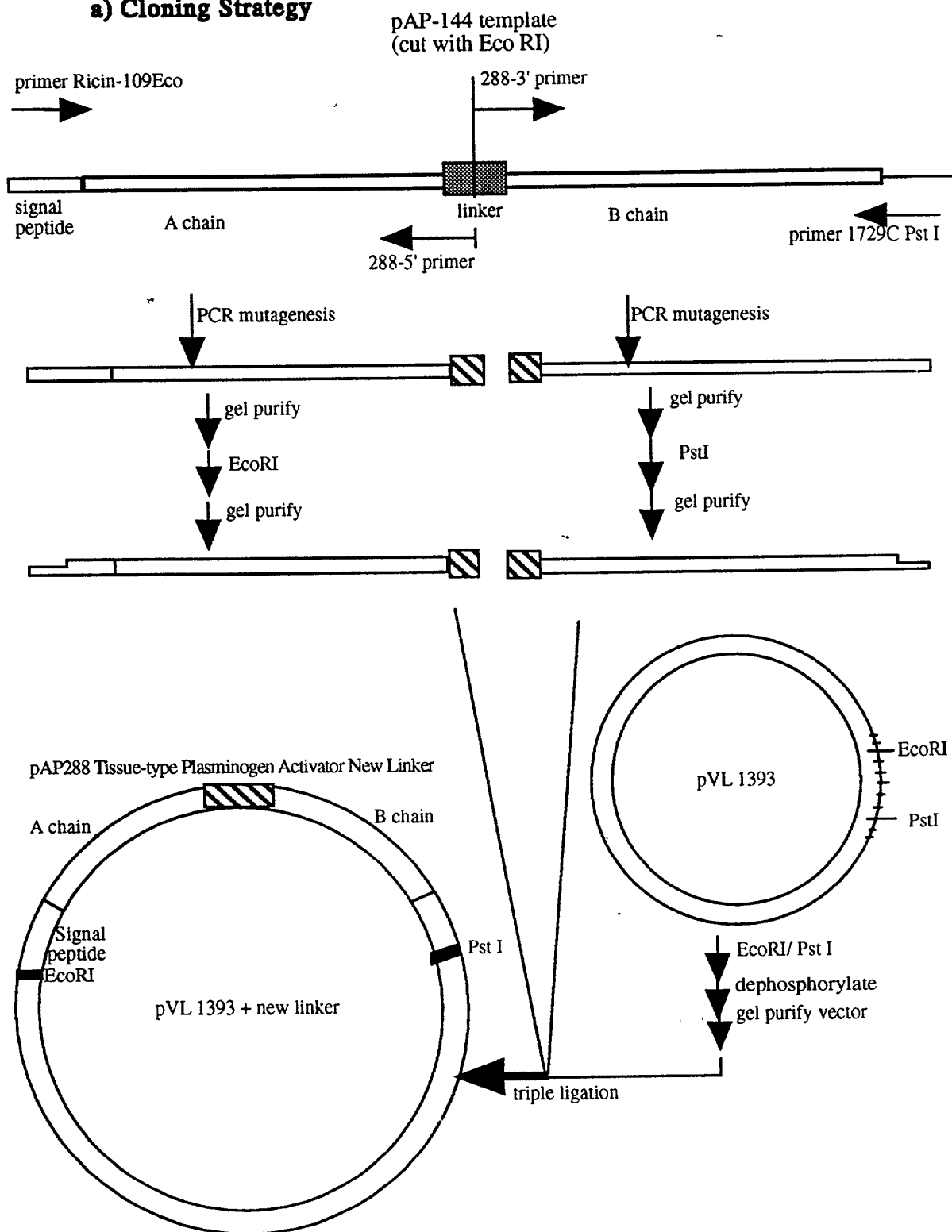
FIGURE 43A**PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393****a) Cloning Strategy**

FIGURE 43B**Sequence of Tissue-type Plasminogen Activator (tPA) Linker Region****WT preprocin linker**

primer 288-3'
 5' - GGTCGTAAAGCTCTTGAAGCTGATGTTTGT -3'
 ***** ** * *

-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
 -----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----
 ***** *****

3' -AGCAGTGTCAAACCGCCTAGACCCGTTTCC -5'
 primer 288-5'

1) PCR mutagenesis

2) Ligate with pVL1393

**pAP 288 linker
(tPA variant)**

-----GGCGGATCTGGGCAAAGG|GGTCGTAAAGCTCTTGAA-----
 -----CCGCCTAGACCCGTTTCC|CCAGCATTTTCGAGAACTT-----

207/254

FIGURE 43C (P1)

Sequence of pAP288 insert

```

          10          20          30          40          50
          |          |          |          |          |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTIONAAGTTTGA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTG

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
```

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208/254

FIGURE 43C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTC AAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGCGGATCTGGGCAAAGGGGTCTGTAAGCTCTTGAAGC
AGCAGTGTCAAACCGCCTAGACCCGTTTCCCAGCATTTTCGAGAACTTCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTTCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCACAACGGAAACGCAATA
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTT CAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGT CAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGA CTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGT CACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGT TACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTT CATACTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

209/254

FIGURE 43C (P3)

GT TTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTAAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGAÇCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATAACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP288

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210/254

FIGURE 43D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Tissue-type Plasminogen Activator (tPA) to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-288 (tPA) linker: A chain- G G S G Q R G R K A L E-B chain

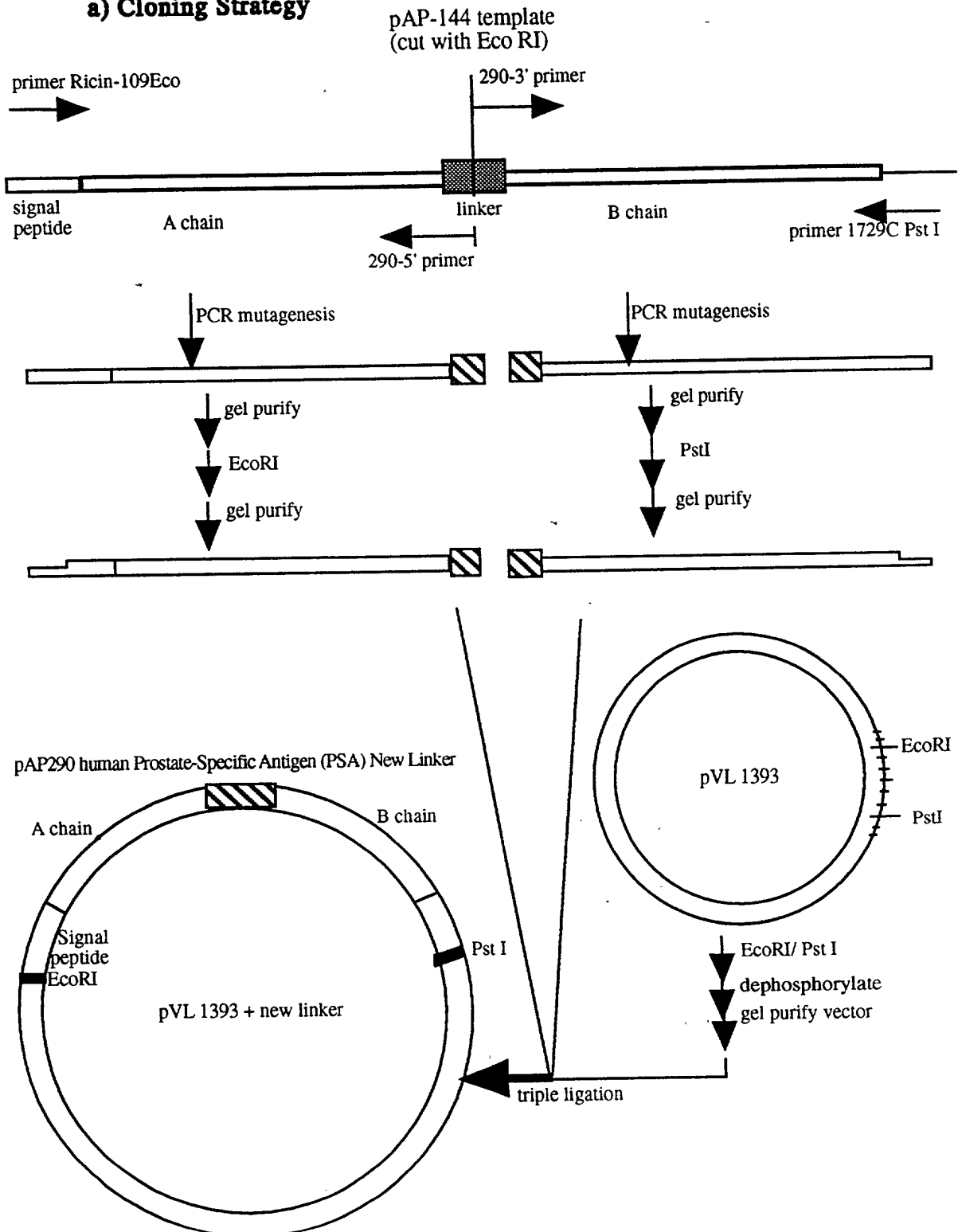
00440 " 04400

211/254

FIGURE 44A

PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy



212/254

FIGURE 44B

Sequence of human Prostate-Specific Antigen (PSA) Linker Region

WT preprocin linker

primer 290-3'

5' - TCTTCCGATATTTTAAATGCTGATGTTTGT -3'

***** *

-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAATTA-----

* ***** *

3' -AGCAGTGTCAAAAGAAACAGTCGAGAAGAG -5'

primer 290-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 290 linker

(PSA variant)

-----TCTTTGTCAGCTCTTCTC|TCTTCCGATATTTTAAAT-----
-----AGAAACAGTCGAGAAGAG|AGAAGGCTATAAAAATTA-----

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213/254

FIGURE 44C (P1)

Sequence of pAP290 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCCACTGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACGGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
```

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214/254

FIGURE 44C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTTTGTCAGCTCTTCTCTCTTCCGATATTTTTAATGC
AGCAGTGTCAAAGAAACAGTCGAGAAGAGAGAAGGCTATAAAAATTACG

951 TGATGTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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215/254

FIGURE 44C (P3)

GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
1751 CTCTTGCAAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP290

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216/254

FIGURE 44D

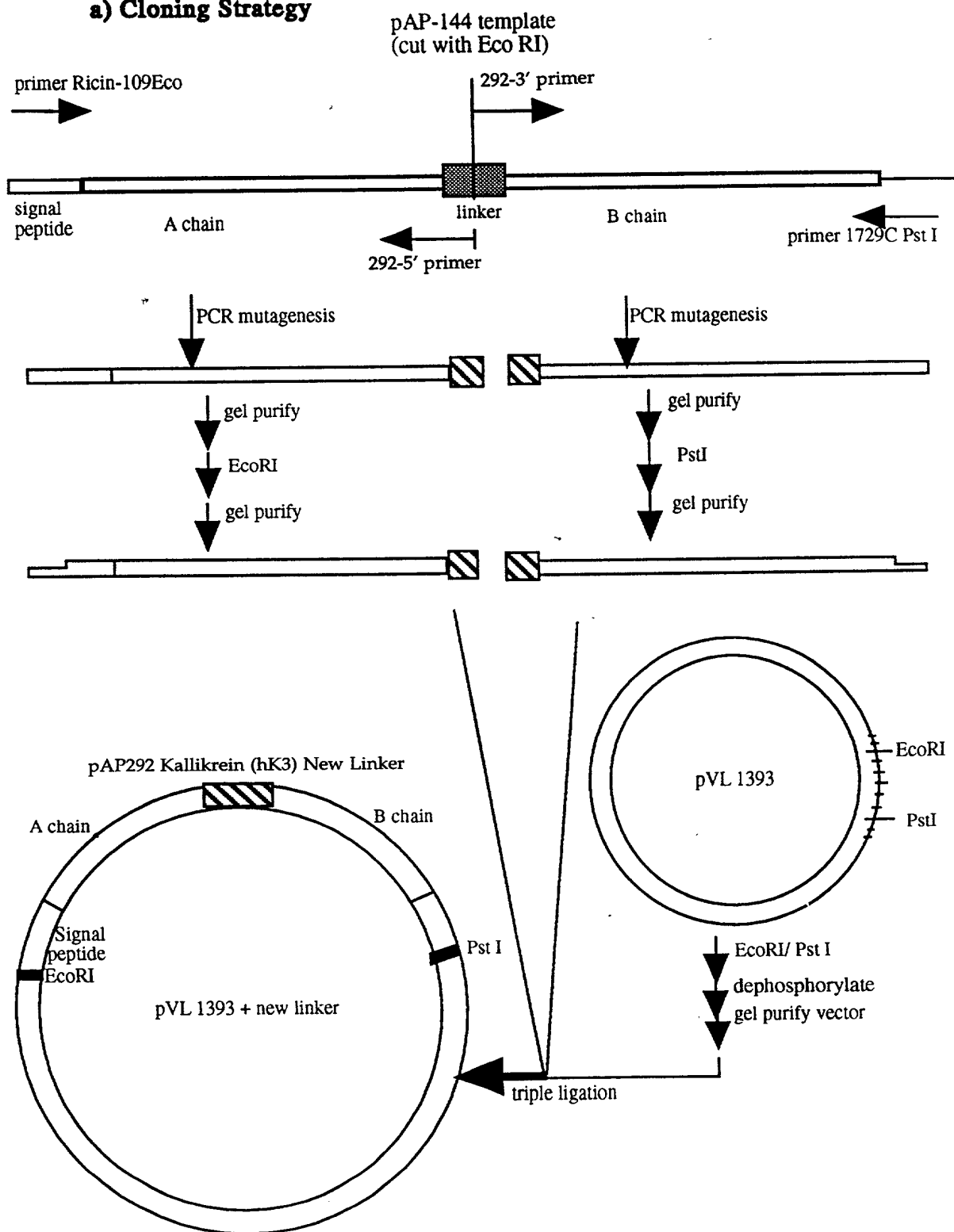
**Amino acid sequence Comparison of Mutant Preproricin Linker
region of human Prostate-Specific Antigen (PSA) to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain
pAP-290 (PSA) linker: A chain- S L S A L L S S D I F N -B chain

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FIGURE 45A

PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy

218/254

FIGURE 45B

Sequence of Kallikrein (hK3) Linker Region

WT preprocin linker

primer 292-3'
5' - ATTATCGGTGGCTTTAATGCTGATGTTTGT -3'
* ** *****
-----TCTTTGCTTATAAGGCCA|GTGGTACCAATTTTAAT-----
-----AGAAACGAATATTCGGT|CACCATGGTTTAAATTA-----
* * *****
3' -AGCAGTGTCAAAAGAAACGGATCTAAATTT -5'
primer 292-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 292 linker
(Kallikrein variant) -
-----TCTTTGCCCTAGATTTAAA|ATTATCGGTGGCTTTAAT-----
-----AGAAACGGATCTAAATTT|TAATAGCCACCGAAATTA-----

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219/254

FIGURE 45C (P1)

Sequence of pAP292 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
   CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTTCTTTATAATTTGCATCCAAATGATTTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTCT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
```

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220/254

FIGURE 45C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT
901 TCGTCACAGTTTTCTTTGCCTAGATTTAAAATTATCGGTGGCTTTAATGC
AGCAGTGTCAAAAGAAACGGATCTAAATTTTAATAGCCACCGAAATTACG
951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAATTATGTCTACGTTTAGTCGAGACCTGAAA
1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG
1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT
1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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221/254

FIGURE 45C (P3)

GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
1751 CTCTTGCA GTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP292

222/254

FIGURE 45D

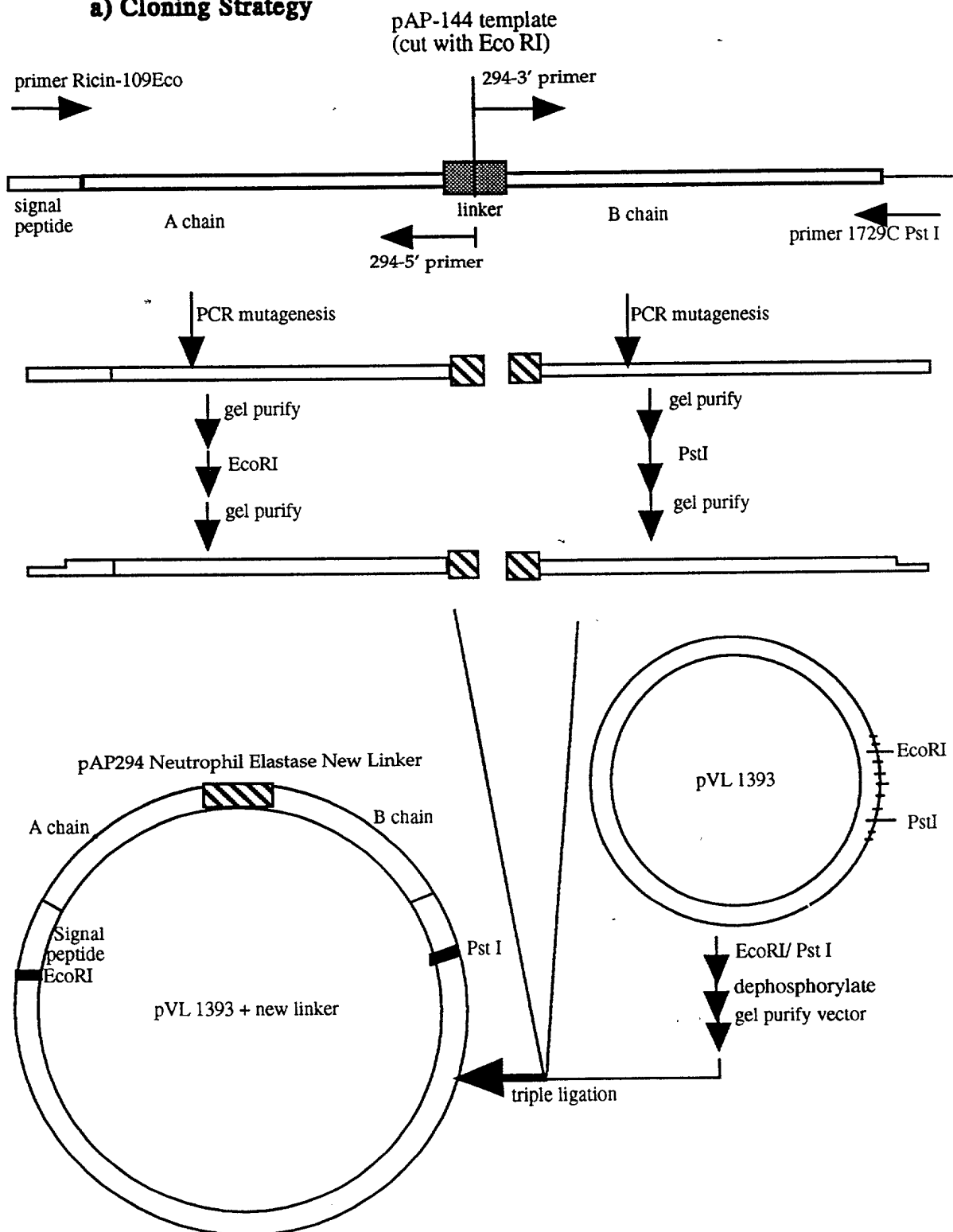
Amino acid sequence Comparison of Mutant Preproricin Linker
region of Kallikrein (hK3) to Wild Type

Wild type ricin linker: A chain- S L L I R F V V P N F N -B chain
pAP-292 (hK3) linker: A chain- S L P R F K I I G G F N -B chain

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FIGURE 46A

PCR Mutagenesis of Preproricin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy

224/254

FIGURE 46B

Sequence of Neutrophil Elastase Linker Region

WT preprocin linker

primer 294-3'

5'- GTTCCTGGTAATTTTAATGCTGATGTTTGT -3'

** *****

-----TCTTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAAATTA-----
*** ** *

3'-AGCAGTGTCAAAAGAAACGAACCGTAACGA -5'

primer 294-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 294 linker

(Neutrophil elastase variant)

-----TCTTTGCTTGGCATTGCT|GTTCCTGGTAATTTTAAT-----
-----AGAAACGAACCGTAACGA|CAAGGACCATTAAAATTA-----

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225/254

FIGURE 46C (P1)

Sequence of pAP294 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACCGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA

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226/254

FIGURE 46C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCCTCT
751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA
801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT
851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGAGGTGGT
901 TCGTCACAGTTTTCTTTGCTTGGCATTGCTGTTCCCTGGTAATTTTAATGC
AGCAGTGTCAAAGAAACGAACCGTAACGACAAGGACCATTAAAATTACG
951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT
1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA
1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC
1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT
1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG
1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG
1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA
1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC
1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT
1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC
1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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227/254

FIGURE 46C (P3)

GTTTTGGCTCTATTAAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
1751 CTCTTGCA GTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
1801 GGACATTGTAAATTTTGTAAGTAAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP294

228/254

FIGURE 46D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Neutrophil elastase to Wild Type**

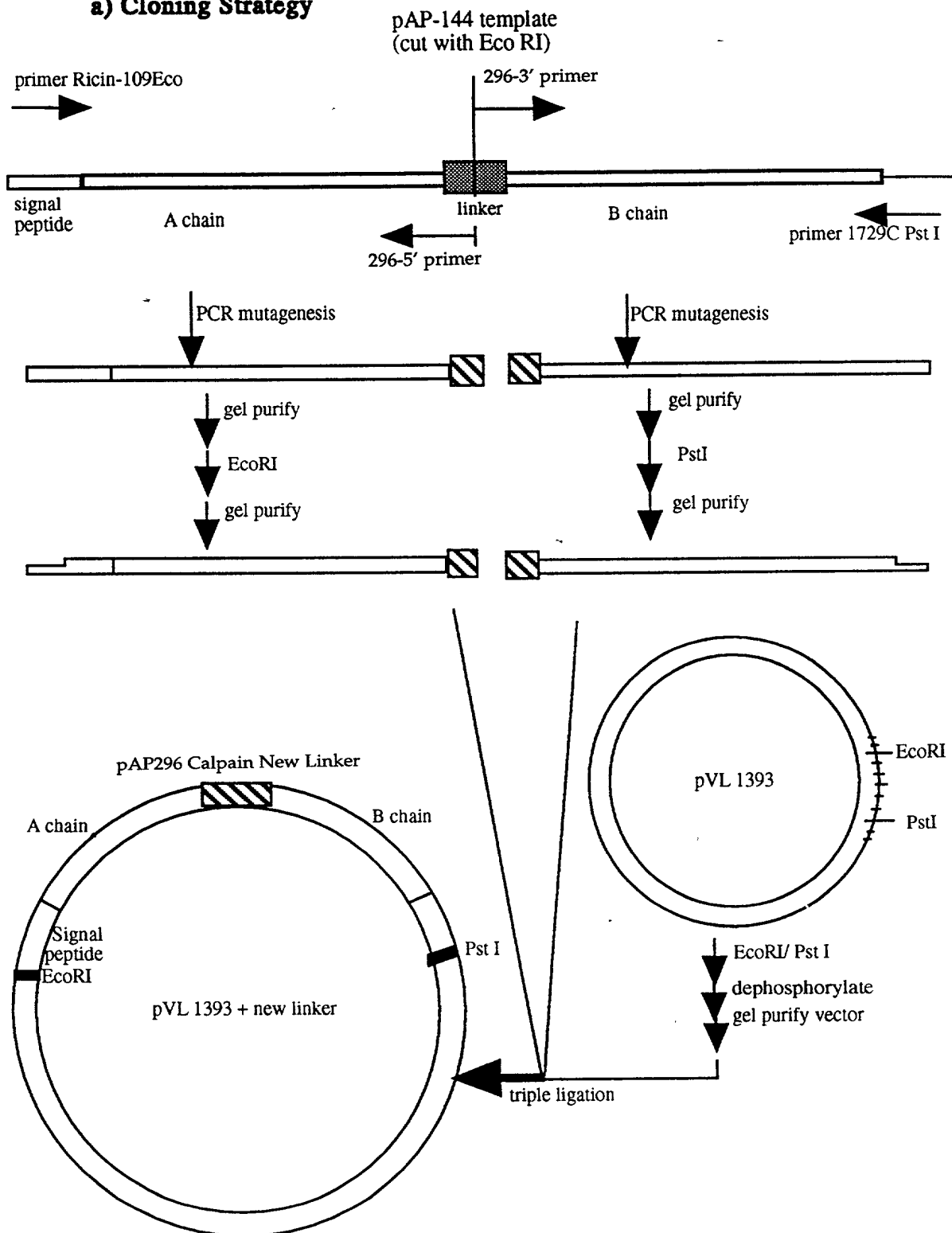
Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

**pAP-294 (Neutrophil elastase) linker:
 A chain- S L L G I A V P G N F N -B chain**

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FIGURE 47A

PCR Mutagenesis of Preprorin Gene to Create A Variant Gene in Baculovirus Transfer Vector, pVL 1393

a) Cloning Strategy

230/254

FIGURE 47B

Sequence of Calpain Linker Region

WT preprocin linker

```
primer 296-3'
5'- ACTCCTAGAACCCCCCAGCTGATGTTTGT -3'
      *****
-----TCCTTGCTTATAAGGCCA|GTGGTACCAAATTTTAAT-----
-----AGAAACGAATATTCCGGT|CACCATGGTTTAAATTA-----
      *   **  *  *****
3'-AGCAGTGTCAAAAAAAGTTTTTATAACAA -5'
primer 296-5'
```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 296 linker
(Calpain variant)

```
-----TTTTTCAAAAATATTGTT|ACTCCTAGAACCCCCCA-----
-----AAAAAGTTTTTATAACAA|TGAGGATCTTGGGGGGGT-----
```

231/254

FIGURE 47C (P1)

Sequence of pAP296 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGATATAACAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT
701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA

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232/254

FIGURE 47C (P2)

CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTTTTTTCAAAAATATTGTTACTCCTAGAACCCCCCAGC
AGCAGTGTCAAAAAAAGTTTTTATAACAATGAGGATCTTGGGGGGGTGCG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAAC TACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT

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233/254

FIGURE 47C (P3)

GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA
1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA
1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA
1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT
1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA
1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT
1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG
1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: PAP296

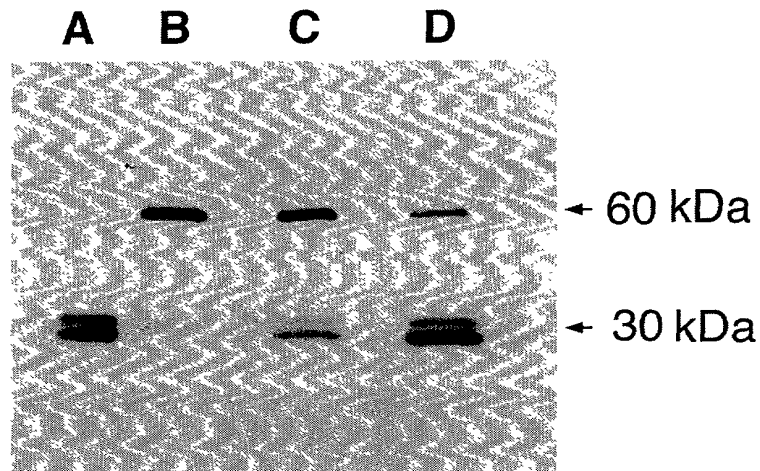
FIGURE 47D

**Amino acid sequence Comparison of Mutant Preproricin Linker
region of Calpain to Wild Type**

Wild type ricin linker: A chain- S L L I R P V V P N F N -B chain

pAP-296 (Calpain) linker: A chain- F F K N I V T P R T P P -B chain

00440" FFF360

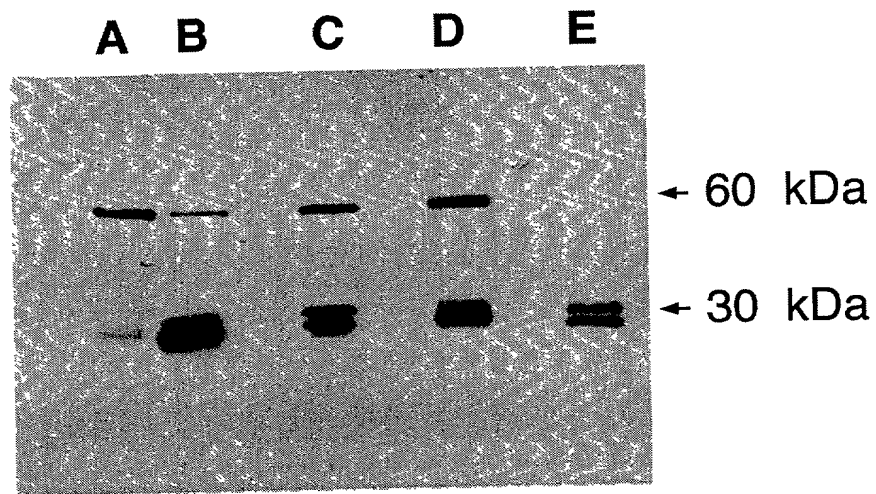
FIGURE 48**Cleavage of pAP 214 by Cathepsin B**

A. Ricin standard

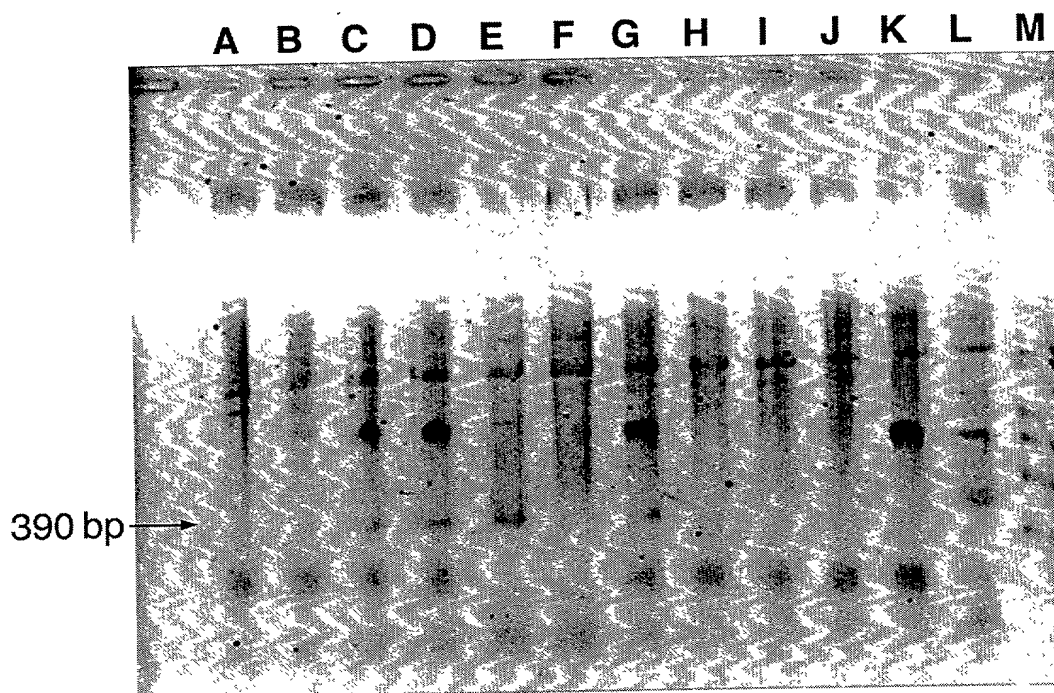
B. pAP 214

C. pAP 214 digested with 100 ng of Cathepsin B (18 hours)

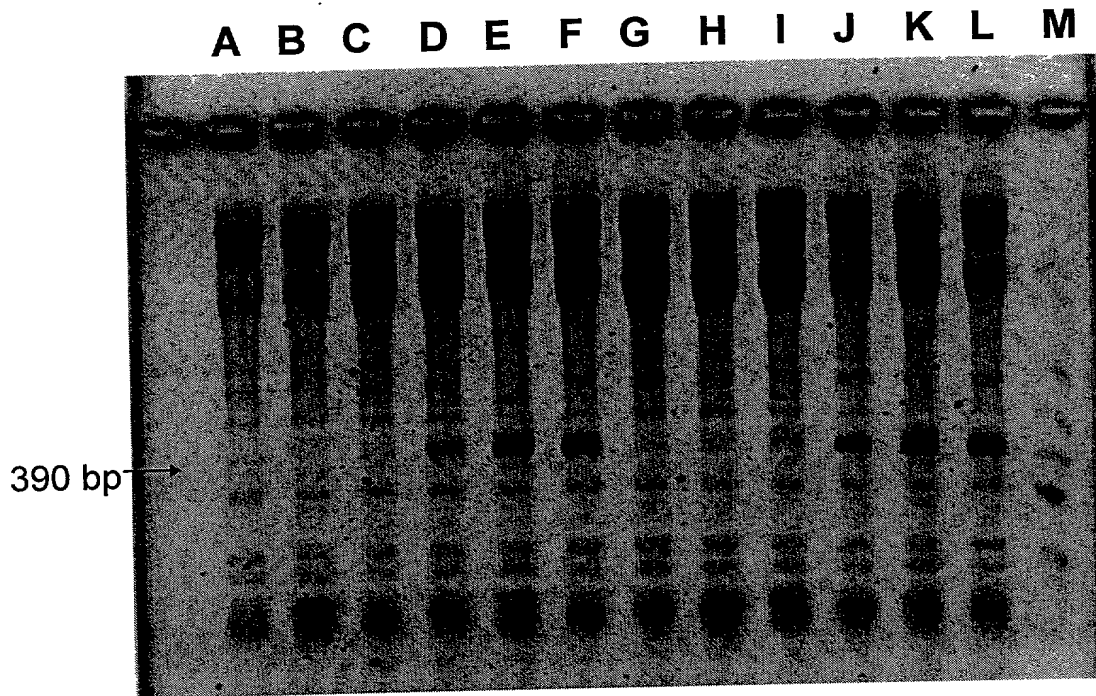
D. pAP 214 digested with 618 ng of Cathepsin B (18 hours)

FIGURE 49**Cleavage of pAP 220 with MMP-9**

- A.** pAP 220
- B.** pAP 220 digested with 200 ng of MMP-9 (16 hrs)
- C.** pAP 220 digested with 20 ng of MMP-9 (16hrs)
- D.** pAP 220 digested with 20 ng of MMP-9 (2hrs)

FIGURE 50**Activation of pAP 214**

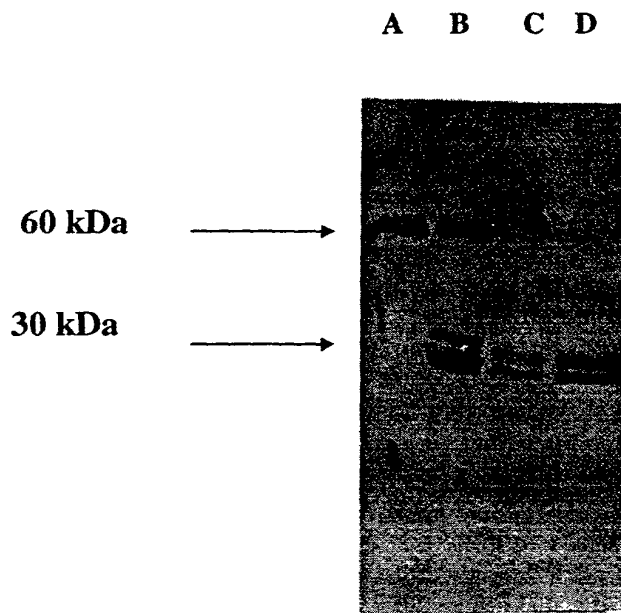
- A.** 41.7 pg of pAP 214 digested with Cathepsin B
- B.** 291 pg of pAP 214 digested with Cathepsin B
- C.** 2.0 ng of pAP 214 digested with Cathepsin B
- D.** 14.2 ng of pAP 214 digested with Cathepsin B
- E.** 100 ng of pAP 214 digested with Cathepsin B
- F.** Negative control
- G.** Ricin A chain
- H.** 41.7 pg of pAP 214 variant
- I.** 291 pg of pAP 214 variant
- J.** 2.0 ng of pAP 214 variant
- K.** 14.2 ng of pAP 214 variant
- L.** 100ng of pAP 214 variant
- M.** RNA ladder

FIGURE 51**Activation of pAP 220**

- A.** 48.5 pg of pAP 220 variant
- B.** 291 pg of pAP 220 variant
- C.** 2.0 ng of pAP 220 variant
- D.** 14.3 ng of pAP 220 variant
- E.** 100 ng of pAP 220 variant
- F.** Ricin A chain
- G.** Negative Control
- H.** 48.5 pg of pAP 220 variant digested with MMP-9
- I.** 291 pg of pAP 220 variant digested with MMP-9
- J.** 2.0 ng of pAP 220 variant digested with MMP-9
- K.** 14.3 ng of pAP 220 variant digested with MMP-9
- L.** 100 ng of pAP 220 variant digested with MMP-9
- M.** RNA ladder

FIGURE 52

Cleavage of pAP-248 Protein by The Human Cytomegalovirus (HCMV) protease



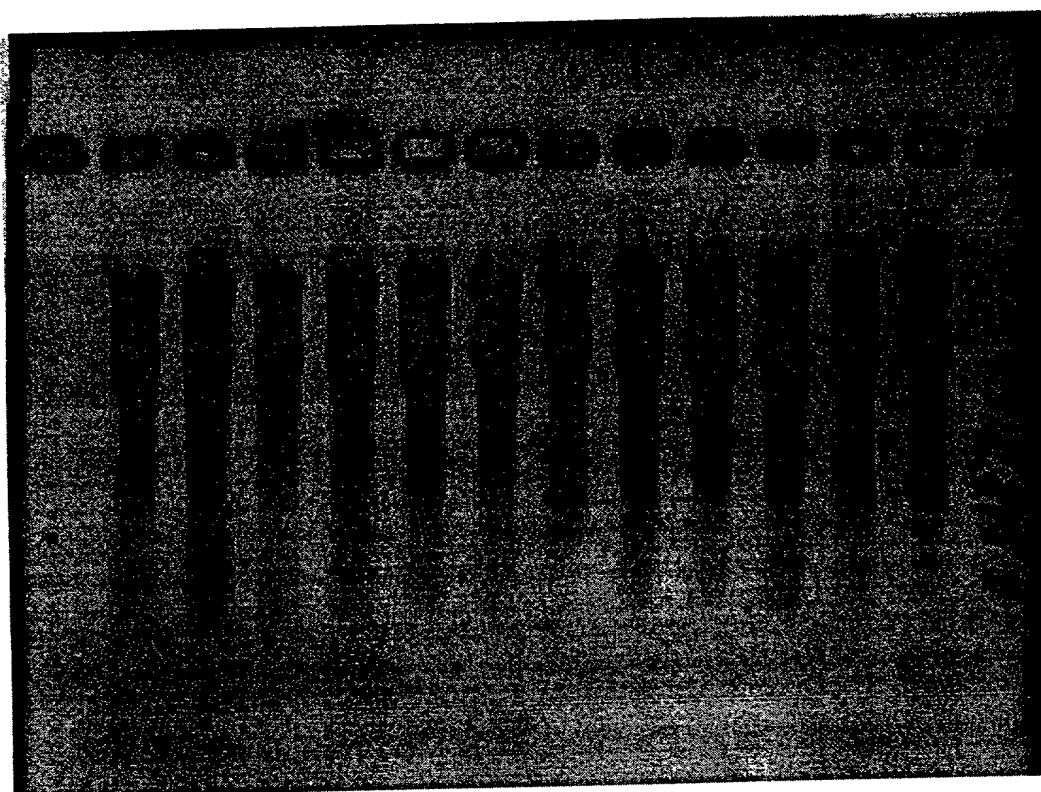
- A. pAP-248 (0.279 μ g)
- B. pAP-248 protein (0.279 μ g) digested with 0.25 μ g of the HCMV protease
- C. Ricin standard (20 ng)
- D. Ricin standard (40 ng)

240/254

FIGURE 53

Activation of pAP-248 Protein

A B C D E F G H I J K L M

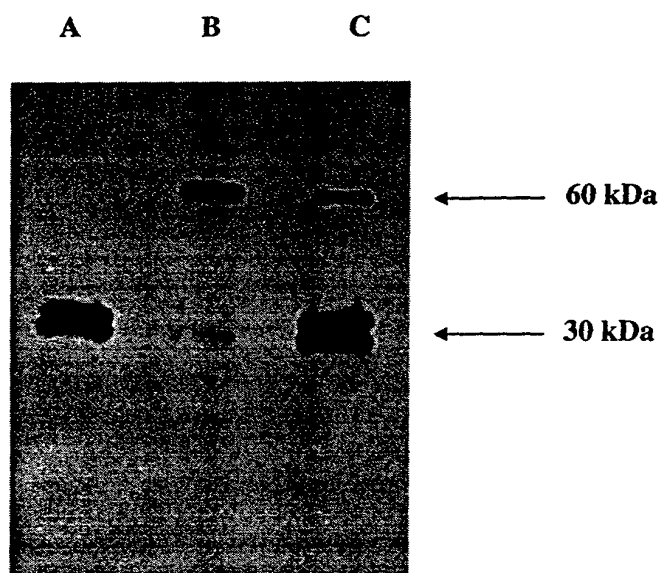


390 b fragment

- A. 90 ng of pAP-248 variant
- B. 12.8 ng of pAP-248 variant
- C. 1.8 ng of pAP-248 variant
- D. 260 pg pAP-248 variant
- E. 37 pg of pAP-248 variant
- F. Negative control
- G. Ricin A chain
- H. 37 pg of pAP-248 digested with HCMV protease
- I. 260 pg of pAP-248 digested with HCMV protease
- J. 1.8 ng of pAP-248 digested with HCMV protease
- K. 12.8 ng of pAP-248 digested with HCMV protease
- L. 90 ng of pAP-248 digested with HCMV protease
- M. RNA ladder

FIGURE 54

Cleavage of pAP-256 protein by The Hepatitis A Virus 3C (HAV 3C) Protease

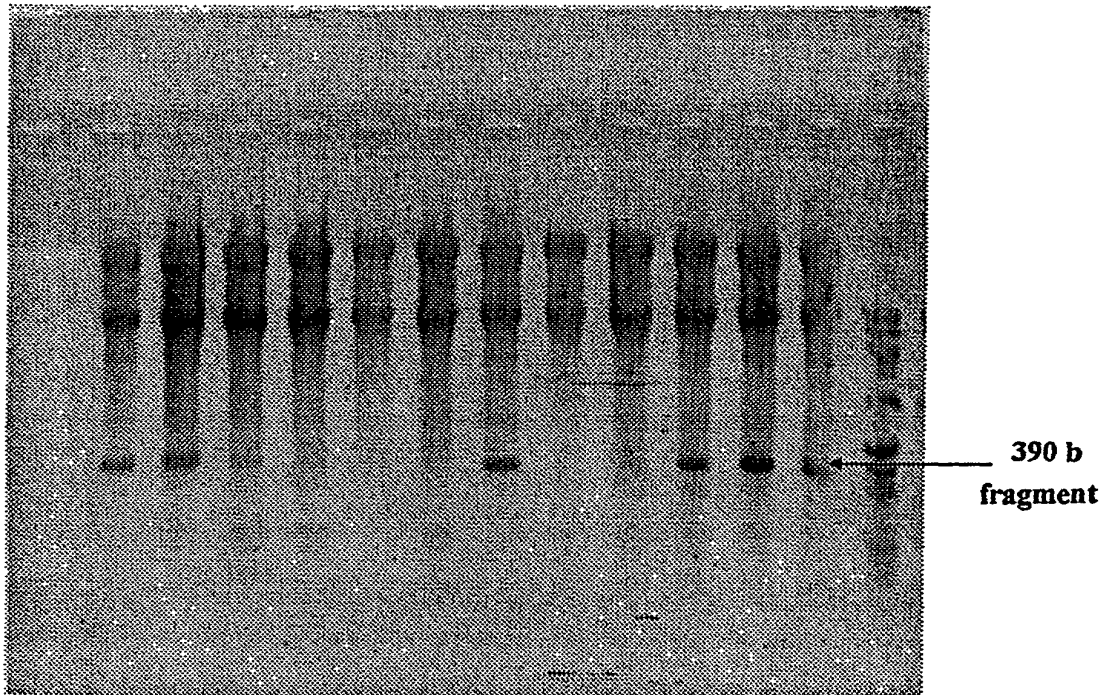


- A. Ricin standard (0.250 ug)**
- B. pAP-256 protein (0.378 ug)**
- C. pAP-256 protein digested (0.302 ug) with 1.25 µg of the HAV 3C protease**

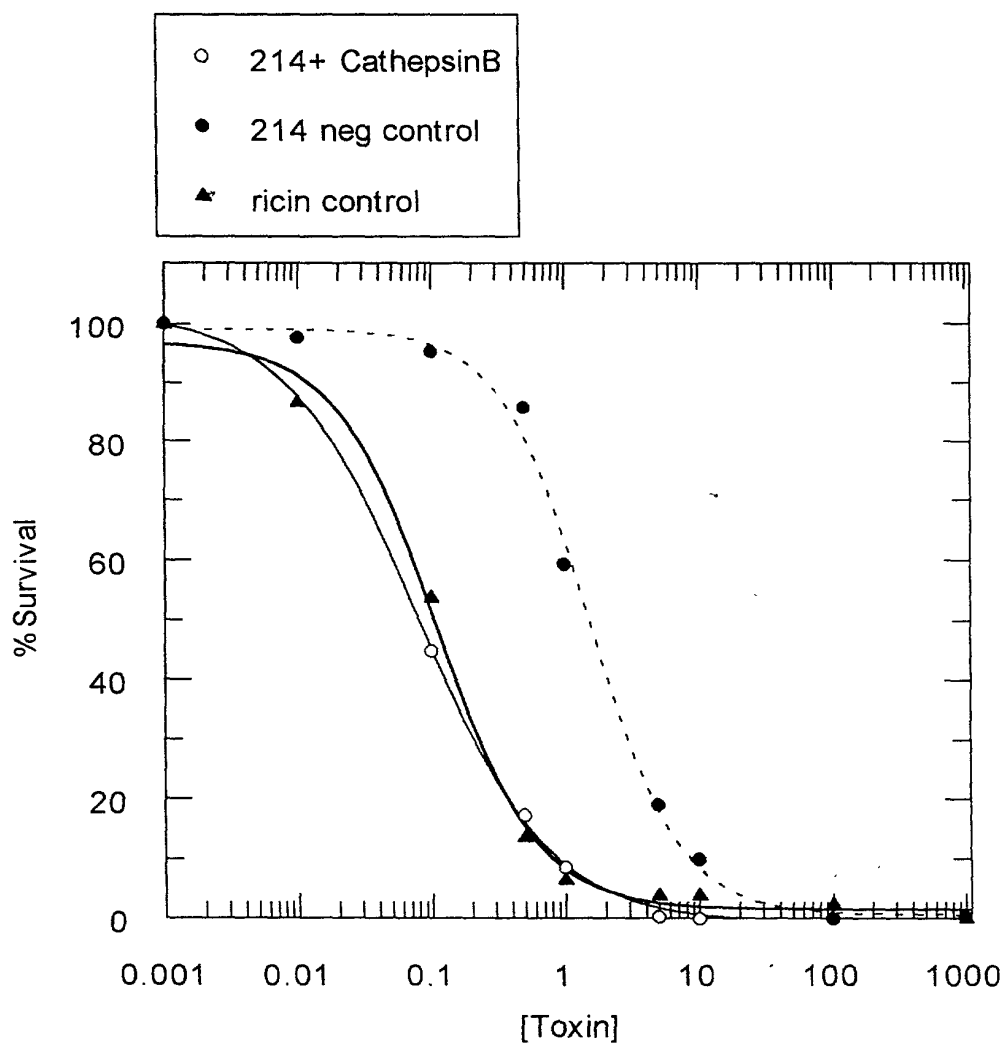
FIGURE 55

Activation of pAP-256 Protein

A B C D E F G H I J K L M



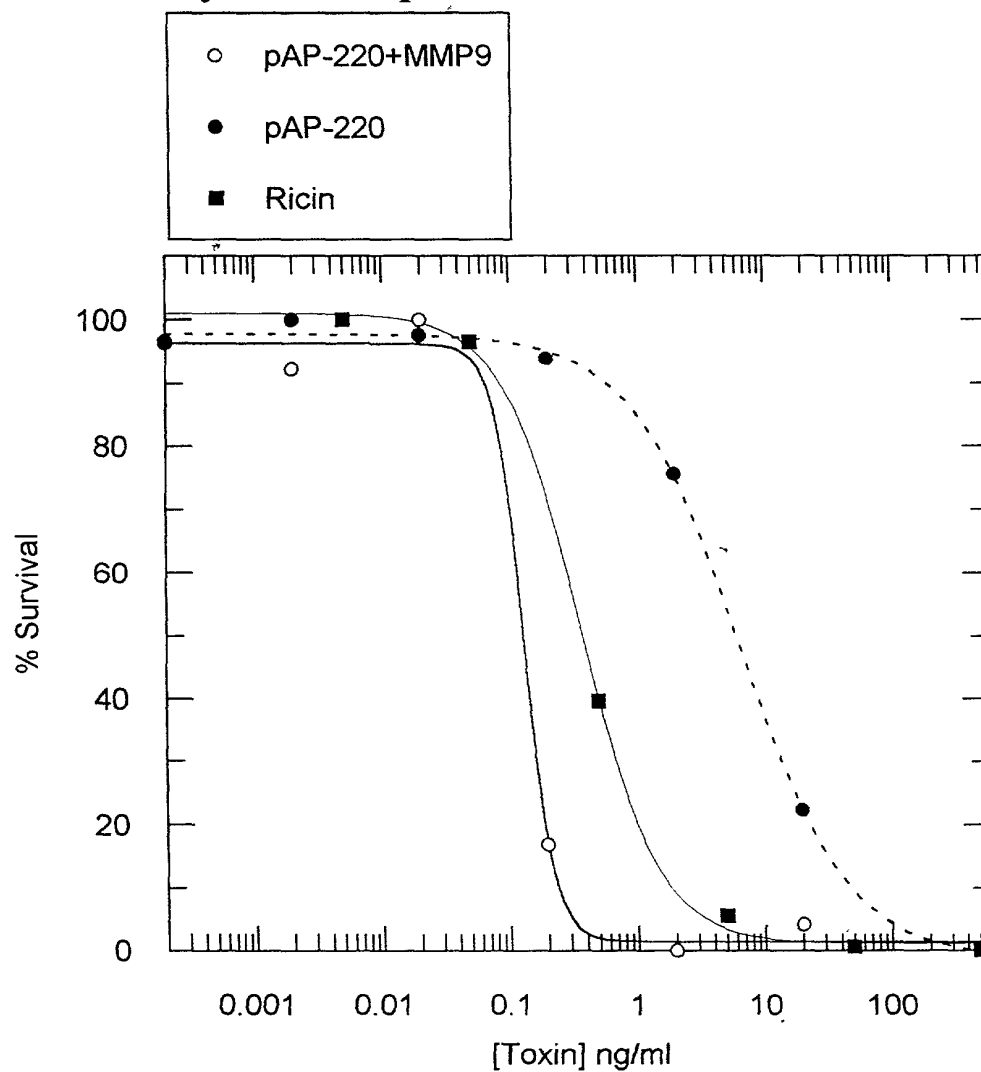
- A. 100 ng of pAP-256 variant
- B. 14.2 ng of pAP-256 variant
- C. 2.0 ng of pAP-256 variant
- D. 291 pg of pAP-256 variant
- E. 41.7 pg of pAP-256 variant
- F. Negative control
- G. Ricin A chain
- H. 41.7 pg of pAP-256 digested with HAV 3C protease
- I. 291 pg of pAP-256 digested with HAV 3C protease
- J. 2.0 ng of pAP-256 digested with HAV 3C protease
- K. 14.2 ng of pAP-256 digested with HAV 3C protease
- L. 100 ng of pAP-256 digested with HAV 3C protease
- M. RNA ladder

FIGURE 56**Cytotoxicity of Digested and Undigested
pAP 214 with Cathepsin B to COS-1 Cells**

	Ricin	pAP 214	pAP 214 + Cathepsin B
IC ₅₀ (ng/ml)	0.11	1.9	0.078
Relative Toxicity	1X	17X	0.7X

FIGURE 57

Cytotoxicity of pAP220 Digested with MMP-9 Compared to Freshly Thawed pAP220 and Ricin on COS-1 Cells

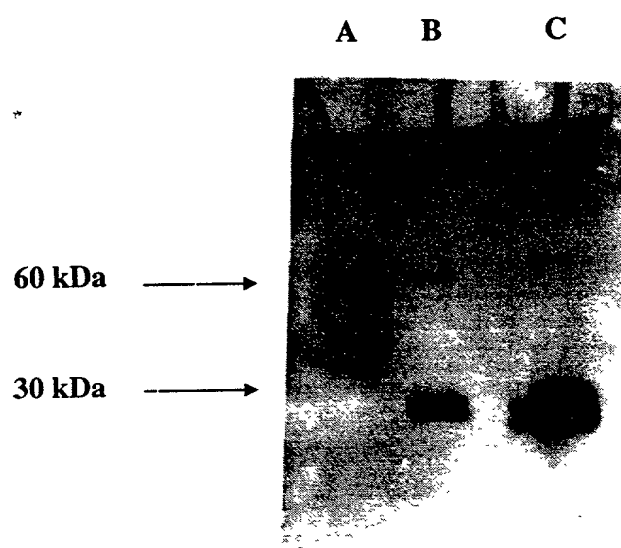


	Ricin	pAP 220	pAP 220 + MMP-9
IC ₅₀ (ng/ml)	0.31	6.7	0.13
Relative Toxicity	1X	22X	0.4X

245/254

FIGURE 58

Cleavage of pAP-270 protein by The Matrix Metalloproteinase 2 (MMP-2)



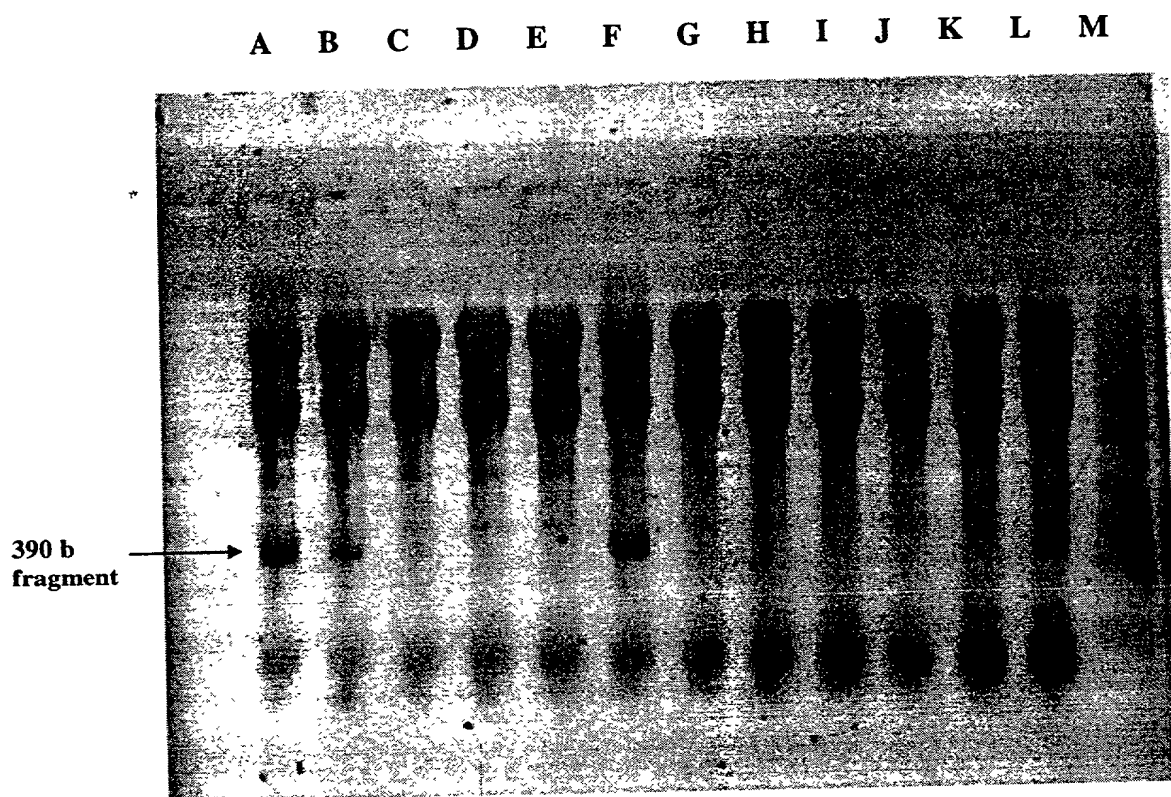
A. pAP-270 (0.120 µg) undigested

B. pAP-270 (0.120 µg) digested with 0.250 µg MMP-2

C. Ricin Standard (0.05 µg)

FIGURE 59

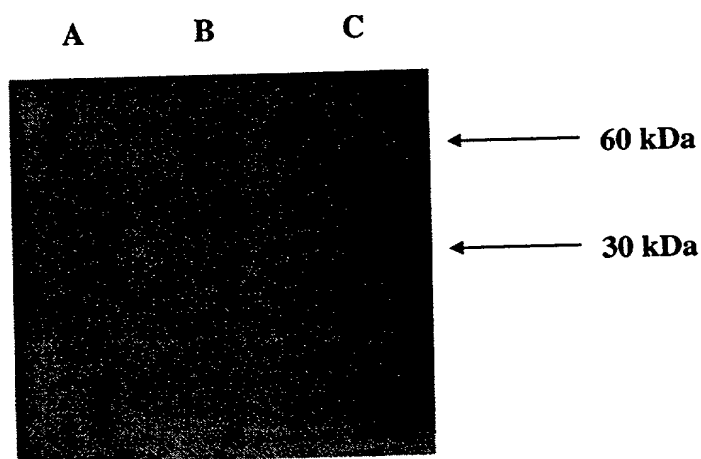
Activation of pAP-270 protein



- A. 100 ng of digested pAP-270
- B. 14.2 ng of digested pAP-270
- C. 2.0 ng of digested pAP-270
- D. 290 pg of digested pAP-270
- E. 46 ng of digested pAP-270
- F. Ricin A chain
- G. Negative control
- H. 46 pg of pAP-270
- I. 290 pg of pAP-270
- J. 2.0 ng of pAP-270
- K. 14.2 ng of pAP-270
- L. 100 ng of pAP-270

FIGURE 60

Cleavage of pAP-288 protein by Plasminogen Tissue Activator (t-PA)



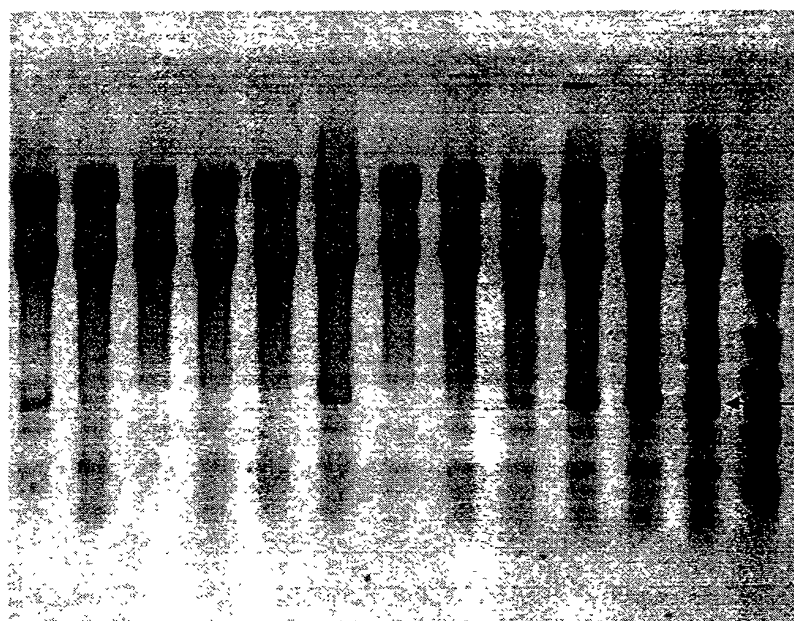
- A. Ricin Standard (0.05 μ g)
- B. pAP-288 (0.66 μ g) undigested
- C. pAP-288 (0.60 μ g) digested with 0.18 μ g of t-PA protease

248/254

FIGURE 61

Activation of pAP-288 protein

A B C D E F G H I J K L M



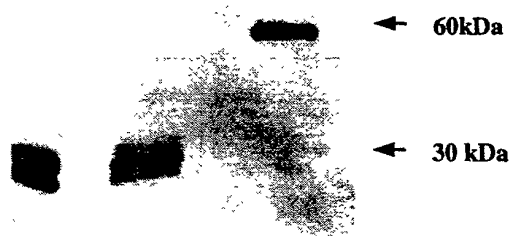
**390 b
fragment**

- A. 200 ng of pAP-288**
- B. 28.4 ng of pAP-288**
- C. 4.0 ng of pAP-288**
- D. 482 pg of pAP-288**
- E. 83.4 pg of pAP-288**
- F. Ricin A chain**
- G. Negative control**
- H. 83.4 pg of pAP-288 digested with tissue Plasminogen Activator (t-PA)**
- I. 482 pg of pAP-288 digested with t-PA**
- J. 4.0 ng of pAP-288 digested with t-PA**
- K. 28.4 ng of pAP-288 digested with t-PA**
- L. 200 ng of pAP-288 digested with t-PA**
- M. RNA ladder**

249/254

FIGURE 62

Cleavage of pAP 294 With Human Neutrophil Elastase

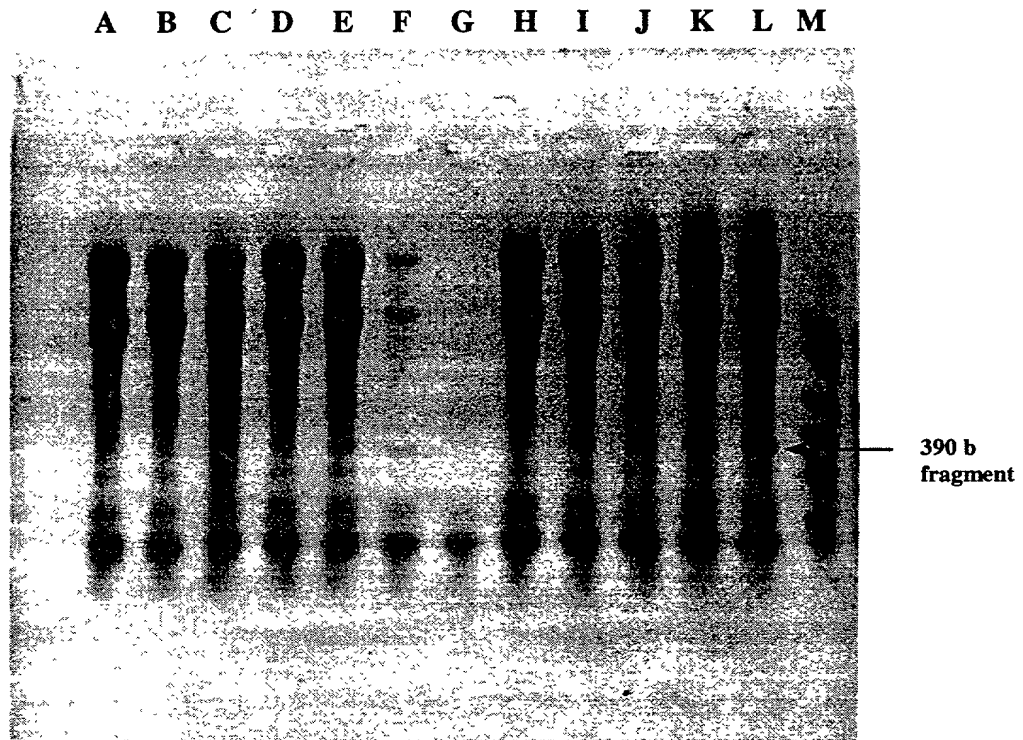


- A. Ricin Standard (0.050 μ g)
- B. pAP 294 protein (0.171 μ g) digested with 1.42 μ g of Human Neutrophil Elastase
- C. pAP 294 protein (0.121 μ g)

250/294

FIGURE 63

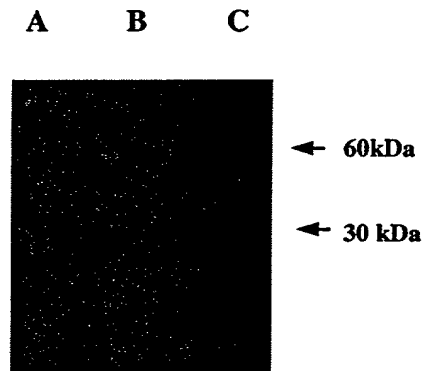
Activation of pAP 294 Protein



- A. 60 ng of pAP 294
- B. 8..57 ng of pAP 294
- C. 1.22 ng of pAP 294
- D. 175 pg of pAP 294
- E. 25 pg of pAP 294
- F. Ricin A chain
- G. Negative Control
- H. 360 ng of pAP 294 digested with Human Neutrophil Elastase
- I. 51 ng of pAP 294 digested with Human Neutrophil Elastase
- J. 7.3 ng of pAP 294 digested with Human Neutrophil Elastase
- K. 1.0 ng of pAP 294 digested with Human Neutrophil Elastase
- L. 150 pg of pAP 294 digested with Human Neutrophil Elastase
- M. RNA ladder

FIGURE 64

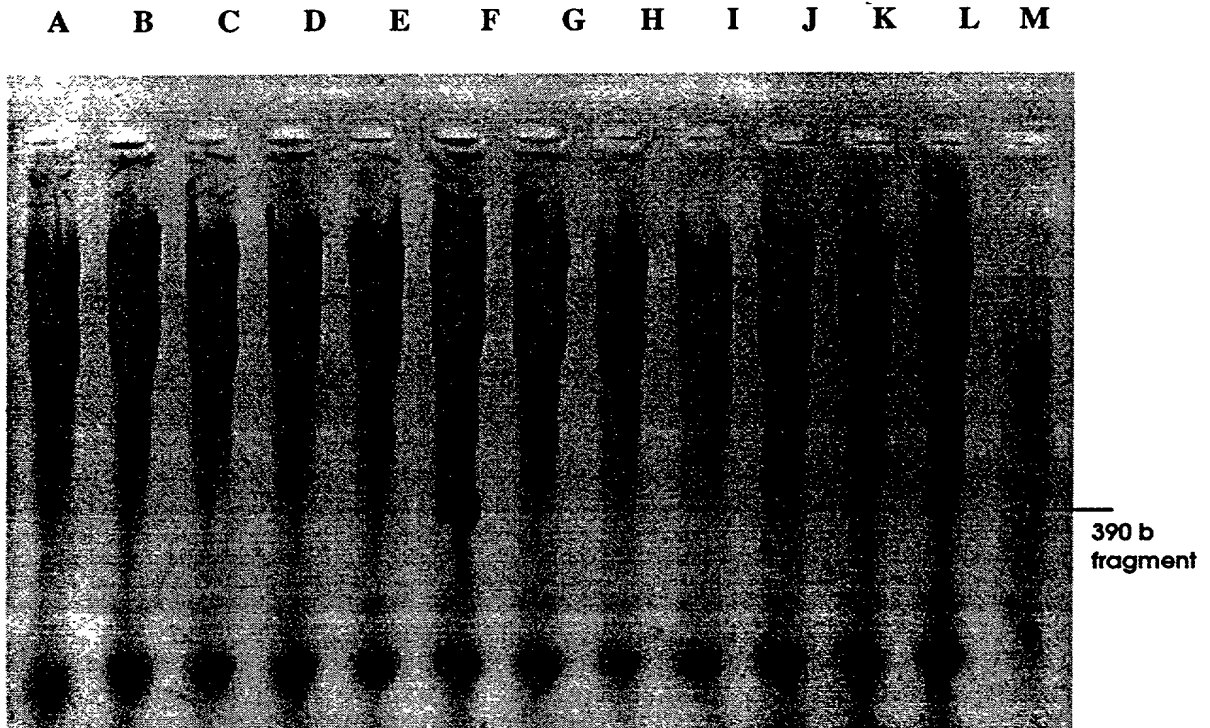
Cleavage of pAP 296 with Calpain



- A. Ricin Standard (0.05 μ g)
B. pAP 296 (0.761 μ g) undigested
C. pAP 296 (0.761 μ g) digested with 4.0 μ g of Calpain

FIGURE 65

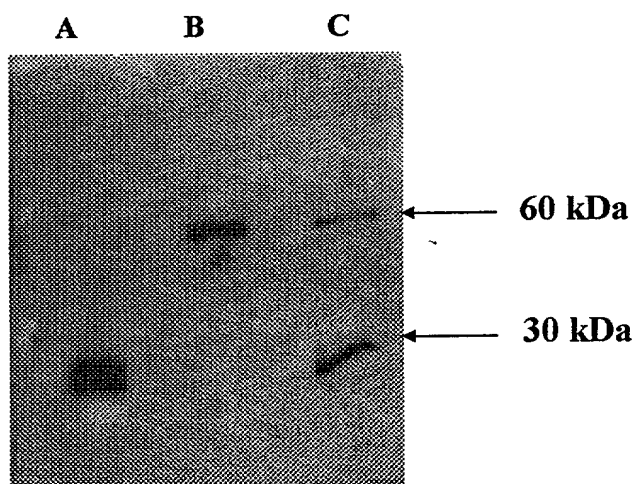
Activation of pAP 296 Protein



- A. 100 ng of pAP 296 variant**
- B. 14.2 ng of pAP 296 variant**
- C. 2.0 ng of pAP 296 variant**
- D. 290 pg of pAP 296 variant**
- E. 46 pg of pAP 296 variant**
- F. Ricin A chain**
- G. Negative control**
- H. 46 pg of pAP 296 variant digested with Calpain**
- I. 290 pg of pAP 296 variant digested with Calpain**
- J. 2.0 ng of pAP 296 variant digested with Calpain**
- K. 14.2 ng of pAP 296 variant digested with Calpain**
- L. 100 ng of pAP 296 variant digested with Calpain**
- M. RNA ladder**

FIGURE 66

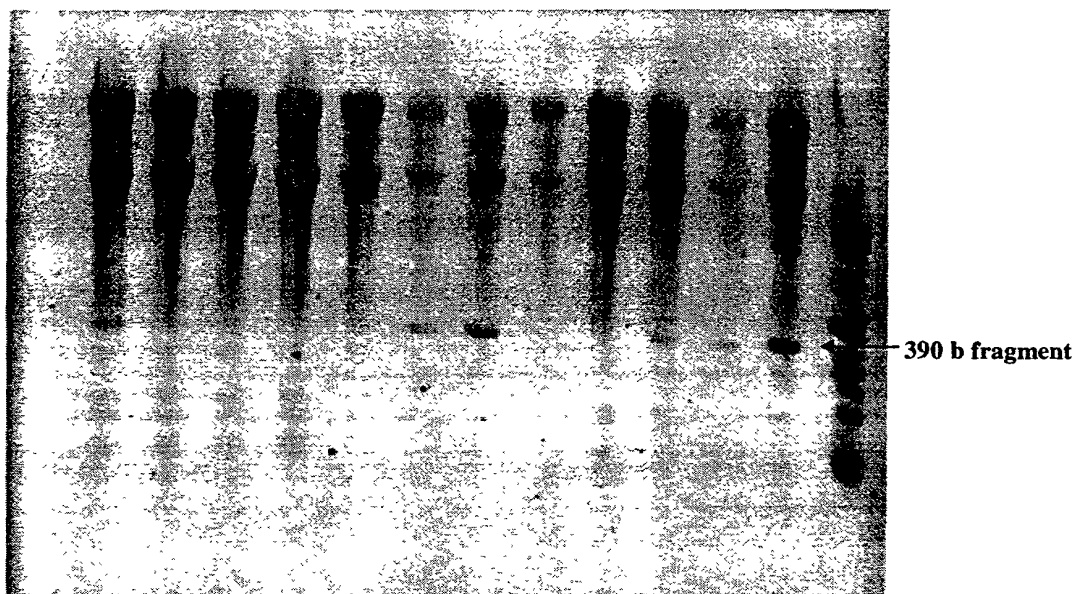
Cleavage of pAP-222 Protein by The Matrix Metalloproteinase 2 (MMP-2)



- A. Ricin Standard (0.250 ug)
- B. pAP-222 Protein (0.250 ug)
- C. pAP-222 protein (0.250 ug) digested with 0.28 ug of MMP-2

FIGURE 67**Activation of pAP-222 Protein**

A B C D E F G H I J K L M



- A. 100 ng of pAP-222 variant
- B. 14.2 ng of pAP-222 variant
- C. 2.0 ng of pAP-222 variant
- D. 291 pg of pAP-222 variant
- E. 41.7 pg of pAP-222 variant
- F. Ricin A chain
- G. Ricin A chain
- H. 41.7 pg of pAP-222 digested with MMP-2
- I. 291 pg of pAP-222 digested with MMP-2
- J. 2.0 ng of pAP-222 digested with MMP-2
- K. 14.2 ng of pAP-222 digested with MMP-2
- L. 100 ng of pAP-222 digested with MMP-2
- M. RNA ladder

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(37 CFR 1.63)**☒ Declaration
Submitted
with Initial
Filing

OR

☐ Declaration
Submitted after Initial
Filing (surcharge
(37 CFR 1.16 (e))
required)

Attorney Docket Number 10447-011

First Named Inventor Thor Borgford

COMPLETE IF KNOWN

Application Number /

Filing Date

Group Art Unit

Examiner Name

As a below named inventor, I hereby declare that

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

RICK-LIKE TOXIN VARIANTS FOR TREATMENT OF CANCER, VIRAL OR PARASITIC INFECTIONS

the specification of which

(Title of the invention)

☒ is attached hereto

OR

☐ was filed on (MM/DD/YYYY)

as United States Application Number or PCT International

Application Number

and was amended on (MM/DD/YYYY)

(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 35(b) of any foreign application(s) for patent or inventor's certificate, or 35(b) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?
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Application Number(s)	Filing Date (MM/DD/YYYY)

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(Page 1 of 2)

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U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
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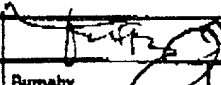
<input checked="" type="checkbox"/> Customer Number 1059		<input type="checkbox"/> Registered practitioner(s) name/registration number listed below	
Name	Registration Number	Name	Registration Number
Denise A. Bereskin	22,257	PC Samuel Frost	31,696
Richard J. Parr	22,256	Philip Mendes de Costa	33,108
M. Roger Hart	25,426	Robert B. Stony	33,108
David W.R. Langton	27,747	John A. Rudolph	36,003
C. Lloyd Barghison	30,246	Robin L.A. Costar	36,016
Timothy J. Bennett	31,083	Michael E. O'Brien	36,026

☒ Additional registered practitioner(s) names on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☒ Customer Number or BAR Code Label  OR ☐ Correspondence address below

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BORGFORD					
Inventor's Signature					Applicant's Date 2/2/00
Residence: City	Burnaby	State	B.C.	Country	Canada
Post Office Address	Apt. 206, 1680 Augusta Avenue				
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City	Burnaby	State	B.C.	ZIP	V5A 2Y6
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